REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

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1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
31-05-2012	Final Report	1-Mar-2009 - 29-Feb-2012

4. TITLE AND SUBTITLE

The Rapid Forensic Evaluation of Microbes in Biodefense research program will develop a rapid screening and detection system for multiple Bio-Threat (BT) agents for the Department of Defense.

5a. CONTRACT NUMBER
W911NF-09-1-0066

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHORS

Dr. Bharathan Narayanaswamy, Dr. Seema Bharathan

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES

IUP Research Institute
IUP Research Institute
1179 Grant Street
Indiana, PA 15701 -

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
55375-CH.3

12. DISTRIBUTION AVAILIBILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT

Global analytical approaches for forensic targets were developed and plant-fungal-viral model system was optimized for double-stranded (ds) RNA and protein signature identification.
This approach developed at Indiana University of Pennsylvania (IUP) utilizes a non bio-threat plant-fungal-viral model system to develop methods and devices for rapid forensic analysis of microbes. The naturally occurring fungus Rhizoctonia solani (a common inhabitant of the soil ecosystem) was used to experiment, develop, optimize

15. SUBJECT TERMS

Rhizoctonia solani, dsRNA, biosensor, proteomics, genomics

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Narayanaswamy Bharathan
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 724-357-5988

Report Title

The Rapid Forensic Evaluation of Microbes in Biodefense research program will develop a rapid screening and detection system for multiple Bio-Threat (BT) agents for the Department of Defense.

ABSTRACT

Global analytical approaches for forensic targets were developed and plant-fungal-viral model system was optimized for double-stranded (ds) RNA and protein signature identification.
This approach developed at Indiana University of Pennsylvania (IUP) utilizes a non bio-threat plant-fungal-viral model system to develop methods and devices for rapid forensic analysis of microbes. The naturally occurring fungus Rhizoctonia solani (a common inhabitant of the soil ecosystem) was used to experiment, develop, optimize and validate bio-threat detection methods and potential device technologies outside of a controlled facility. This model system has proven to be an economical approach to knowledge acquisition that does not require an elaborate and more complex biological safety level (BSL) 3 capabilities. The fungus also harbors double stranded RNA viruses that influence the parasitic and saprobic activity of the fungus and could be potentially exploited for bioterrorism. Since the project's initiation in February 2009, significant progress has been made specifically: A rapid manufacturing protocol was developed; 13 different viral agents were characterized for their nucleic acid (milestone 1) and targetable proteins (milestone 3); universal primer for BT detection with double-stranded (ds) RNA genome has been identified and verified. The sequencing of the R. solani genome in partnership with the J. Craig Venter Institute (JCVI) is complete. So far 16 R. solani proteins have been annotated and several mutants with reduced genome complement (RGC) have been generated. Protocols for the integration of modern imaging methods into bio-defense research to elucidate mechanisms at the macro level that characterize host-microbe interactions were developed (milestone 2). Techniques used include panoramic imaging, confocal imaging, and LC/MS/MS technologies for protein signature development.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

CHEMICAL AND BIOLOGICAL DEFENSE SCIENCE AND TECHNOLOGY CONFERENCE

Paper Topic # 1).

Microbial Interactions ---Panoramic Imaging and Cytology of Non-Self Fungal Fusions in the Pathogenic Fungus Rhizoctonia solani Mark Smith1, Anthony G. Gutierrez2, Andrea Strein1, Seema Bharathan1, Marc Cubeta3, Shan Min Chin1, Chin Hong Siew1, Richard Somiari4 and Narayanaswamy Bharathan1 1Indiana University of Pennsylvania, Indiana, Pennsylvania, 15705; 2 The U..S. Army Institute of Public Health, Aberdeen Proving Ground, MD, 21010; 3Center for Integrated Fungal Research, North Carolina State University, Raleigh, NC 27606; 4ITSI-Biosciences, Johnstown, Pennsylvania 15901, USA

The integration of modern imaging methods into bio-defense research will help elucidate mechanisms at the macro level that characterize host-microbe interactions. Our research at Indiana University of Pennsylvania funded by Defense Threat Reduction Agency (DTRA) in cooperation with ITSI-Biosciences and Department of Health uses fungal-viral model system to study microbial interactions; cytoplasmic exchange of genetic elements; protein factors; and the underlying causes of host cell death. In the present study using 19 different paired combinations, we observed hyphal interactions between compatible and incompatible Rhizoctonia solani isolates using a compact portable macro image stacking device (patent pending) that allows one to photograph objects as small as 0.5 mm using a standard DSLR camera. When the mycelia of R. solani encounter mycelia with a different genetic background, distinct barrage lines (BL) form. Several ultra-high resolution micro/macro images were generated showing high degree of stratification near or close to the tip of the fungal mycelium suggesting active protein and lipid synthesis. The device uses a DSLR camera with a 1-5x Macro lens. Since the lens focuses on a depth of field 1/10th to 1/50th of the width of the image it frames, a stacking device was developed earlier (Macropod tm , USAIPH) to capture multiple images in depth to compile in to a single fully focused image. The Macropod tm consists of a macro lens equipped DSLR which is mounted fixed on a 3 legged stand such that the DSLR is optically in line with one leg. That leg carries a specimen mount which can be moved with precision short distances along its length, the Z axis. The specimen mount itself can also move the specimen in the X and Y axes for shorter distances for stitching panoramic shots. The Z axis stack can be downloaded to a computer and stacked using any number of stacking algorithms. Edited images provided extra high resolution that allowed us to observe perfect hyphal anastomosis in compatible pairs. The hyphae never anastomosed with incompatible pairs (i.e., the hyphae remained parallel or crossed over without merging). These behaviors appear to result from the detection of one or more diffusible factors or due to up-take of viruses, toxins and traffic within fungal cells. Our results also suggest that the attraction to other hyphae in pairs of incompatible isolates was increased by supplementation of the growing medium with activated charcoal. We will report data concerning programmed cell Death (PCD) with one or two approaching hyphae. Techniques used include panoramic imaging, immunofluorescence, confocal imaging, and LC/MS/MS technologies for protein signature development.

Funding was provided by a grant from Department of the Army and Defense Threat Reduction Agency (DTRA) to Dr. N. Bharathan and Dr. Seema Bharathan. Award # W911NF-09-1-0066 PROPOSAL # 55375CH

Global Analytical Approaches to Forensic Target Optimization in a Plant-Fungal-Viral Model System. Seema Bharathan1, Richard Somiari2, Shan Min Chin1, Chin Hong Siew1, Florentina Mayko, Stephen Russell2, Andrea Strein1, Doreen Domingo1, and Narayanaswamy Bharathan1, Indiana University of Pennsylvania, Indiana, Pennsylvania, 15705; 2ITSI-Biosciences, Johnstown, Pennsylvania 15901, USA

Paper Topic # 2).

Global Analytical Approaches to Forensic Target Optimization in a Plant-Fungal-Viral Model System. Seema Bharathan1, Richard Somiari2, Shan Min Chin1, Chin Hong Siew1, Florentina Mayko, Stephen Russell2, Andrea Strein1, Doreen Domingo1, and Narayanaswamy Bharathan1, Indiana University of Pennsylvania, Indiana, Pennsylvania, 15705; 2ITSI-Biosciences, Johnstown, Pennsylvania 15901, USA

ABSTRACT The “Rapid Forensics Analysis of Microbes” program at Indiana University of Pennsylvania in collaboration with ITSI-Biosciences utilizes plant and fungal viruses to develop methods and devices for rapid forensic detection of microbes. These organisms are not classified as biothreat (BT) agents and hence it was possible to conduct experiments in a regular laboratory and develop models that could be applied to other areas of BT agent research. Our focus was to identify extra-chromosomal RNA and proteins that are characteristic of each fungal model system. The secondary focus was -- to determine if the presence of viruses can be detected in these plant and fungal models and see-- if the presence of these viruses in any way causes detectable changes in protein expression. Specifically, this program utilizes the plant pathogenic fungus Rhizoctonia solani as a model. R. solani is highly virulent on potato and other economically important chlorophyllous plant species; it is naturally occurring with one phenotype but with multiple genotypes harboring different viruses. In fact some R. solani carry more than one type of virus. The representative variants of the wild (heterokaryon) type fungal strains and several reduced (homokaryon) complement (RC) strains of R. solani were characterized and their potential targets identified at the molecular level for the presence of extra-chromosomal double-stranded (ds) RNA and proteins. Briefly, about 11 dsRNA belonging to 11 different R. solani isolates that included both wild type and RC strains were cloned and sequenced. A unique strategy was

developed for amplification and cloning different middle (M)-size dsRNA fragments by synthesizing random primer-dN6 (5'-CCTGAATTCGGATCCTCCNNNNNN-3'). Full-length PCR products representing several genomic segments were cloned into p-Drive cloning vector (Qiagen) and sequenced. The sequences were determined using Sanger sequencing with an ABI 3730 sequencer (Retrogen Inc. San Diego, CA). The sequences were compared for similarity against the non-abundant sequence databases of EMBL, EU, using the BLAST algorithm. Several clones were picked; preliminary sequence comparison of some mycoviral dsRNA suggests 26-30% sequence identical to coat protein of Carrot Cryptic Virus, Beet Cryptic Virus, and the capsid protein of the White Clover Cryptic Virus. In order to test for sensitivity and specificity, probes were prepared from cloned dsRNA fragments of the wild-type dsRNA and hybridized to dsRNA and cloned DNA from both the other wild-type and RC strains of R. solani. Probes were dual labeled (5'FAM/3'BHQ-1) and the design for qPCR assays was done using Biosearch Technologies (Novato, CA) Real Time Design™ software. Thus, our discovery platform for the first time has developed a protocol to generate PCR products from unknown dsRNA sequences belonging to the genome of pleomorphic viruses infecting the R. solani isolates that are heterokaryon and a homokaryon. Preliminary assessment of the identified proteins indicate that some are unique to each model, and represent candidate targets that will allow the selective detection of the target with high sensitivity and specificity.

Funding was provided by a grant from Department of the Army and Defense Threat Reduction Agency (DTRA) to Dr. N. Bharathan and Dr. Seema Bharathan. Award # W911NF-09-1-0066 PROPOSAL # 55375CH

Paper Topic # 3).
Detection of Tomato Mosaic Virus (ToMV) Using One Step Reverse Transcription PCR Alan Kessler**, Charles Voyton*, Shan Min Chin**, Chin Hong Siew**Dr. Seema Bharathan**, Dr. N. Bharathan** *Biochemistry Program, **Department of Biology, Indiana University of Pennsylvania, Indiana, Pennsylvania 15705 Annual Undergraduate Research Conference, Indiana University of Pennsylvania April4, 2012.
This poster won the award for the best student Presentation in the College of Natural Sciences and Mathematics.

Tomato Mosaic Virus (ToMV) is a single stranded RNA virus belonging to the Tobamo Virus Group. The genome of the virus is single-stranded (ss) RNA about 6.2-kilobase (kb) in length and that is highly infectious. The virus is transmitted by mechanical injury and there is no known invertebrate vector that has been found to transmit the virus in nature. There are reports that the virus is spread across the continent via fog, mist, clouds, springs, and all forms of flowing water. The decrease in spruce and hemlock population in the Northern Alleghany forests may be caused by shifting environmental factors that make these trees more susceptible to attack. A project was undertaken to specifically identify the distribution of the ToMV in the aquatic habitat in order to predict the distribution of the virus in the Western Alleghany Forest. Total RNA was extracted from various sources including streams, ponds, virus infected soil water, and plant tissue infected with the virus. Presence or absence of the Viral RNA was tested using one-step-reverse transcription (RT)-PCR, in which the combination of degenerate deoxinosine (dI)-substituted primers were designed to amplify part of the polymerase region of the ToMV viral RNA genome, followed by a nested PCR amplification that increased specificity and sensitivity of detection. An attempt was made to alleviate the problems of generic detection and identification of ToMV RNA by nested RT-PCR using DI-containing primers. Preliminary results from real-time detection of ToMV RNA from various sources suggest the presence of copious amount of ToMV associated viral RNA in soil water, including the streams. Results from the study to increase specificity and sensitivity of detection using virus ToMV viral RNA specific probes indicate that RNA as high as 79.0µg/mL and as low as 3.4µg/mL could be detected.

Number of Presentations: 3.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
2011/08/30 1(	2	1. Seema Bharathan, Richard Somiari, Shan Min Chin, Chin Hong Siew, Jessica Richards, N. Bharathan , 2. Deanna Belsky, Jon Henninger, Chin Hong Siew, Shan Min Chin, Seema Bharathan, N. Bharathan, 3. Jonathan Henninger, Deanna Belsky, Chin Hong Siew, Shan Min Chin, Seema Bharathan, N. Bharathan. 1. Characterization of RNA and proteins signatures from viruses to develop single platform devices for multiple detection of pathogens 2. Centrifugal Analysis of the Cytoplasmic Fractions of the Plant Pathogenic Fungus 3. PCR Cloning of Double-Stranded (ds) and Single-Stranded (ss) RNA from Viral Agents for Signature Biodetection, 1. Department of Life Science, National Institute of Technology, Roukela, India (08 2011)
2010/08/31 1'	1	1. Deanna Belsky, Jon Henninger, Chin Hong Siew, Shan Min Chin, Seema Bharathan and N. Bharathan 2. Jonathan Henninger, Deanna Belsky, Chin Hong Siew, Shan Min Chin, N. Bharathan and Seema Bharathan. 1. Centrifugal Analysis of the Cytoplasmic Fractions of the Plant Pathogenic Fungus Rhizoctonia solani for Double-Stranded RNA (dsRNA) 2. PCR Cloning of Double-Stranded (ds) and Single-Stranded (ss) RNA from Viral Agents for Signature Biodetection, (04 2011)

TOTAL: 2

Number of Manuscripts:

Books

Received	Paper

TOTAL:

Patents Submitted

None

Patents Awarded

None

Awards

Dr. Bhararathan Narayanaswamy Principal investigator in the project received the

i) Outstanding Research Award for the outreach sponsored Research program at Indiana University of Pennsylvania (IUP), Indiana, Pennsylvania (IUP), Indiana, PA 15705 (2010-11)

ii) Received the Life Time Achieving Award 2011-12 for excellent service to students and Scholarship at IUP (2011-12).

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Anjan H. Bangalore	0.10	
Haripriya G. Patil	0.10	
Shruthi Shivashankar	0.10	
Mehak Mahajan	0.10	
Shan Min Chin	0.33	
Chin Hong Siew	0.33	
Doreen Domingo	0.10	
FTE Equivalent:	**1.16**	
Total Number:	**7**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Seema Bharathan	0.20	
Narayanaswamy Bharathan	0.20	
FTE Equivalent:	**0.40**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Louis Colaini	0.30	Chemistry
Alan Kessler	0.30	Biology
Charles Voyton	0.30	Biochemistry
FTE Equivalent:	**0.90**	
Total Number:	**3**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	3.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:	3.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:	3.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	3.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
Ms. Kristy Anthony	0.20
Ms. A. Rao	0.10
Dr. Doreen Domingo	0.15
FTE Equivalent:	**0.45**
Total Number:	**3**

Sub Contractors (DD882)

1 a. J. Craig Venter Institute

1 b. J. Craig Venter Institute
9704 Medical Center Drive
Rockville MD 208503433

Sub Contractor Numbers (c):
Patent Clause Number (d-1):
Patent Date (d-2):
Work Description (e):
Sub Contract Award Date (f-1):
Sub Contract Est Completion Date(f-2):

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1 a. J. Craig Venter Institute

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9704 Medical Center Drive
Rockville MD 208503433

Sub Contractor Numbers (c):
Patent Clause Number (d-1):
Patent Date (d-2):
Work Description (e):
Sub Contract Award Date (f-1):
Sub Contract Est Completion Date(f-2):

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1 a. Integrated Technologies & Services International-Biosciences

1 b. 633 Napoleon Street
Johnstown PA 15901

Sub Contractor Numbers (c):
Patent Clause Number (d-1):
Patent Date (d-2):
Work Description (e):
Sub Contract Award Date (f-1): 5/1/2010 12:00:00AM
Sub Contract Est Completion Date(f-2): 2/1/2011 12:00:00AM

Inventions (DD882)

Scientific Progress

See Attchment.

Technology Transfer

Final Report

Rapid Forensic Evaluation of Microbes in Biodefense

Foreword

The "Rapid Forensics Analysis of Microbes" program utilizes plant, fungal and viruses to develop methods and devices for rapid forensic analysis of microbes. By using microorganisms that are not classified as biothreat agents as models, it is possible to conduct experiments and develop models that can be applied to other areas. Specifically this program utilizes the fungus *Rhizoctonia solani* as a model because it is a naturally occurring fungus with one phenotype but multiple genotypes because it harbors different viruses. In fact some *R. solani* carry more than one type of virus. This means that using this model organism it will be possible to evaluate the extent to which it will be possible to discriminate between the fungus carrying for example dsRNA from a plant carrying single stranded (ss) RNA. A major challenge for developing better and faster ways for detection of biological agents of national interest is the limited availability of suitable facilities and equipment to many researchers, as well as the difficulty in getting security/safety clearances that will allow many more researchers to work with biothreat agents. A way around this problem is the use of model organisms that are not regarded as biothreat agents. Using model organisms that are not pathogenic provides an opportunity to experiment, develop, optimize and validate methods and devices in a regular laboratory prior to testing the hypothesis and/or performance of the protocol/device with a bio-threat agent of interest in a controlled facility. In three years, representative variants of the model plant and fungal strains were characterized, and potential molecular targets identified at the nucleic acid and protein levels.

Contents

Milestone 1: Characterization of fungal and viral nucleic acids. This milestone will achieve the goal of full characterization of viruses infecting *R. solani* and tomato mosaic virus RNA with the aim of identifying nucleic acid signatures that can be used to discriminate between the viral and fungal models.

Milestone 2: Characterization of fungal and viral proteins. This milestone will achieve the goal of performing proteomic analysis of *R. solani* and tomato mosaic virus proteins with the aim of identifying protein signatures that can be used to discriminate between the viral and fungal models.

Milestone 3: Explore the feasibility of sequencing the genome of *R. solani* AG-1/AG-4. This milestone will achieve the goal of setting the stage for potential sequencing of the genome of *R. solani* AG-1/AG-4. This will allow the development of a peptide database to allow a more efficient and accurate annotation of *R. solani* AG-1/AG-4 at the molecular level.

Milestone 1: Characterization of fungal and viral nucleic acids. This milestone will achieve the goal of full characterization of viruses infecting *R. solani* and tomato mosaic virus RNA with the aim of identifying nucleic acid signatures that can be used to discriminate between the viral and fungal models.

TASK 1: Purification and Characterization of Plant viral nucleic acids **Tomato Mosaic Virus (ToMV) single stranded (ss) RNA.**

Experimental Protocols

Project Design and Experimental Plan

i) Viral genomic RNA from Tomato Mosaic Virus (ToMV) was isolated from partially purified virus and infected tissue. Isolation of RNA from Tomato Mosaic Virus (ToMV) ssRNA: High quality ssRNA were purified as described below using combination of clarification, tissue disruption, followed by low-speed and high speed centrifugation. These step-wise processes were performed on ice and under aseptic conditions.

ii) Extraction of ssRNA from partially purified viral agent. Purified ToMV was diluted 10-fold in 2X NET (0.2M NaCl, 20mM Tris, 2mM EDTA and 10% SDS. The mixture was heat to 70ºC in hot ddH2O to disrupt virus structure and RNA extracted by phenol extraction. The aqueous phase was treated with 2.5 volume of ethanol (EtOH). The concentration and purity of ssRNA was assessed using spectrophotometric analysis and tested for its integrity, purity and concentration; analyzed by gel electrophoresis. Eventually the RNA was used for generic detection by nested PCR.

iii) a) **Concentration of RNA**

More accurate estimation of nucleic acid concentration was made using UV spectroscopy. DNA, RNA, and protein strongly absorb ultraviolet light in the 260 to 280 nm range. Nucleic acids absorb strongly at 260 nm and less strongly at 280 nm while proteins just do the opposite. The general rules for determining concentrations of nucleic acids at 260 nm are:

1 OD unit of single-stranded RNA is 40µg/ml

Concentration [RNA] µg/ml = $OD_{260\ nm}$ X Dilution factor X 40 (Extinction Coefficient). The results are shown in Appendix A and Figure 1

b) **RT-PCR Analysis Tomato Mosaic Virus (ToMV):** One-step-RT-PCR protocol was tested and validated, using combination of degenerate deoxinosine (dI)-substituted primers (Table 1) . These Primers were designed to amplify part of the polymerase region of the ToMV viral RNA genome, followed by a nested PCR amplification step to increase specificity and sensitivity of detection.

cDNA Synthesis using Degenerate primers Reverse Transcription PCR

Reverse transcription (RT) PCR of both the purified ssRNA from ToMV was done using the Ambion Retroscript© Kit with few modifications.

ssRNA or ToMV were denatured at 97°C for 90 seconds in the presence of 1% DMSO and random decamers or degenerate primers Appendix A and Table 1 were used.

All primers were synthesized by Invitrogen.

Denatured ssRNA was subjected to RT-PCR as described below:

2µL 10X RT Buffer

4 µL dNTP mix

1 µL RNase Inhibitor

1 µL of MMLV-RT Reverse Transcriptase to a final volume of 20µL.

Bio-Rad MyCycler Thermocycler was programmed for the following:

44°C for 1 hr, followed by 92°C for 10 min. The newly generated cDNA-RNA hybrids were used PCR. immediately for the 2nd round PCR

Conventional PCR of cDNA of ToMV ssRNA

Conventional PCR of the cDNA from the RT-PCR was conducted using Ambion SuperTaq™ Plus DNA Polymerase.

The reaction was set up as follows:

5µL of RT-PCR reaction,

5µL of 10X PCR Buffer (100 *mM* Tris-HCl pH 9.0, 500 *mM* KOAc, 15 *mM* MgSO4),

2.5µL dNTP mix (2.5 *mM* each dATP, dCTP, dGTP, dTTP),

33.5µL of nuclease- free water,

5 *µM* of primers (TOBRTUP1 (GAGTACGCIGCITICAGAC, where I denotes deoxyinosine), and TOBRTD02 (CGCTTCAAAGTTCCA) for ToMV ssRNA; and 1 U of SuperTaq DNA polymerase.

The reactions were placed in a Bio-Rad MyCycler programmed for the following:

Initial denaturation at 94°C 4 minutes,

30 cycles of denaturation (94°C for 30 seconds), annealing (46°C for 30 seconds), and extension (72°C for 4 minutes), followed by a final extension of 72°C for 10 minutes.

PCR products were analyzed by 1% agarose- gel electrophoresis in 1X TAE buffer. Agarose-gels were run at 37 V/cm for 2 to 3 hours.

Gel electrophoressis Pictures Results are in Appendix A

Real-time PCR of cDNA of TomV ssRNA

The cDNA from the RT-PCR reaction was done using DNA Master SYBR Green I from Roche in an Eppendorf RealPlex© Real Time PCR Thermocycler. The reactions set up included

13.6µL of nuclease- free water,

0.4µL of MgCl2 stock (25 *mM*),

5 μM each of the primers TOBRTD02 and TOBRTUP1 (for ToMV),

2µL of the SYBR Green Master Mix and 2µL of template from the previous RT-PCR reaction.

The reactions were placed in the thermocycler programmed for the following: initial denaturation at 94°C for 3 minutes,

40 cycles of denaturation (94°C for 15 seconds),

annealing (46°C for 15 seconds), and

extension (72°C for 1 minute), followed by a

final extension at 72°C for 5 minutes. Fluorescence readings were taken at the extension phase of each cycle, and the samples were ultimately subjected to a melting curve analysis.

RESULTS

The partially purified RNA was tested for its integrity, purity and concentration; analyzed by gel electrophoresis. Eventually the RNA was used for generic detection by nested PCR. The results are presented below:

a) **Concentration of RNA**

More accurate estimation of nucleic acid concentration was made using UV spectroscopy. DNA, RNA, and protein strongly absorb ultraviolet light in the 260 to 280

nm range. Nucleic acids absorb strongly at 260 nm and less strongly at 280 nm while proteins just do the opposite. The general rules for determining concentrations of nucleic acids at 260 nm are:

1 OD unit of single-stranded RNA is 40μg/ml
Concentration [RNA] μg/ml = $OD_{260\ nm}$ X Dilution factor X 40 (Extinction Coefficient).
The results are shown in Figure 1 (Appendix A).

b) **Qualitative Assessment of RNA by Agarose Gel Electrophoresis Denaturing Agarose Gels** (MOPS –Formaldehyde gels)

a. Method: Since RNA is single-stranded, most RNA can form secondary structures via intra molecular base pairing. It was therefore essential that denaturing gel is used to separate the RNA. RNAse free reagents will be used, to optimize your results. A typical protocol for denaturing RNA gels is taken from "Current protocols in Molecular Biology". Electrophoretric Gel analysis of RNA (Appendix A Figure 2).

c) **RT-PCR Analysis:** The microbial forensic project incorporated one-step-RT-PCR , in which the combination of degenerate deoxinosine (dI)-substituted primers were designed to amplify part of the polymerase region of the ToMV viral RNA genome, followed by a nested PCR that amplification that increased specificity and sensitivity of detection. In the present study, an attempt was made to alleviate the problems of Generic Detection and Identification of ToMV RNA by nested RT-PCR using DI-containing primers. The list and design of the degenerate primers are shown in table 1.

The higly degenerate primers (Tobamo 1 and Tobamo 2) were designed from conserved motifs of the RdRp genes of tobamoviruses (Gibbs *et al.*, 1998). All primers were synthesized by Integrated DNA technologies and Invitrogen. The purified PCR products were cloned into p-Drive cloning vector (Figure 5) and transformed into *E. coli* competent cells according to the manufacturer instructions (Qiagen PCR Cloning Kit). The transformed plasmids were purified from transformed *E. coli* cells by using GeneJEt Plasmid Miniprep Kit (Fermentas). The transformation efficiency was determined to be 4.6%. In all 22 clones were selected and the purified plasmids were cut by EcoRI FastDigest Enzyme (Fermentas) and ran them on the gel to determine the sizes of the cloned fragments (Figure 6). The sizes of the cloned fragments are shown in the Table 4.

All these cloned plasmids containing the insert were eventually sequenced Retrogen Inc. The sequences were compared for similarity against non-redundant sequence database of the National Center for Biotechnology Information, Bethesda, USA or the EU Consortium of Molecular Biology http://www.ebi.ac.uk/Tool/sss/fasta/. The sequence comparison to known Tobamovirus groups have shown 90% sequence alignment Figure 7. Under the optimized amplification conditions, nested RT-PCR assays using leaf extracts as templates also produced expected of the ToMV isolates (Figure 2). This type of generic RT-PCR method can have very important practical implications in DNA microarray DNA technology, by providing high quality amplified DNA targets tio be hybridized onto a micro-array based plant-virus cDNA chip. Using the system we have developed with degenerate primers it is possible to detect many different viruses in mixed infections. These protocols support our effort to demonstrate multi-platform effectiveness for our biodetector. The preliminary results from primer design, probe design (Figures 8 and 9) are shown in Table 8 and FAM profile in Figure 10.

b) **Design of Dual labeled oligonucleotide probes for ToMV detection:** Genomic assays for ToMV viral RNA will be tested using profluorescent oligonucleotide probes that are covalently labeled at the 5‘ end with a fluorophore at the 3‘ end with a quencher. The probes will be designed using software from Integrated DNA Technologies (IDT) for the following two targets of the ssRNA agent RNA-directed RNA polymerase and the Coat protein (Cp) gene of the TMV virus.

Probe design for the target sequence is shown in Figure 1 below and the principle of Dual labeled probes is shown in Figure 2. These allow achieving maximum specificity and sensitivity. All real-time PCR assays will be done using eppendorf Mastercyler^{R} ep Realplex with optimized qPCR in small reaction volumes. Probe design for the target sequence is shown in Figure 10 below and the principle of Dual labeled probes is shown in Figure 11. These allow achieving maximum specificity and sensitivity. All real-time PCR assays will be done using eppendorf Mastercyler^{R} ep Realplex with optimized qPCR in small reaction volumes.

A probe specific for the sequence of interest is used in PCR together with specific PCR primers (see below). This probe is designed to anneal between the PCR primers. During

the extension phase of PCR, the 5'-3' exonuclease activity of Taq DNA polymerase cleaves the fluorescent reporter from the probe. The amount of free reporter accumulates as the number of PCR cycles increases. The fluorescent signal from the free reporter is measured in real time and allows quantification of the amount of target sequence.

Probes Design:

i) It was specific to the ToMV RNA replication; **RNA-directed RNA polymerase**: for the following Amino acid Sequence

(NCBI Resources http://www.ncbi.nlm.nih.gov/nuccore/AJ417701)

LKYYTVVMDP LVSIIRDLER VSSYLLDMYK VDAGTQQQLQ VDSVFKNFNL FVAAPKTGDI SDMQFYYDKC LPGNSTLLNN YDAVTMKLTD ISLNVKDCIL DMSKSVAAPK DVKPTLIPMV RTAAEMPRQT GLLENLVAMI KRNFNSPELS GVVDIENTAS LVVDKFFDSY LLKEKRKPNK NFSLFSRESL NRWIAKQEQV TIGQLADFDF VDLPAVDQYR HMIKAQPKQK LDLSIQTEYP ALQTIVYHSK KINAIFGPLF SELTRQLLDS IDSSRFLFFT RKTPAQIEDF FGDLDSHVPM DVLELDISKY DKSQNEFHCA VEYEIWRRLG LEDFLAEVWK QGHRKTTLKD YTAGIKTCLW YQRKSGDVTT FIGNTVIIAS CLASMLPMEK LIKGAFCGDD SLLYFPKGCE YPDIQQAANL MWNFEAKLFK KQYGYFCGRY VIHHDRGCIV YYDPLKLISK LGAKHIKDWD HLEEFRRSLC

ii) Coat protein (Cp) gene of the ToMV capsid protein (ENA|CAD22082|CAD22082.1 Tomato mosaic virus capsid protein)

atgtcttactcaatcacttctccatcgcaatttgtgtttttgtcatctgtatgggctgaccctatagaattgttaaacgtttgtacaaactc
gctaggcaaccagtttcaaacacagcaagcaagaactactgttcaacagcagttcagcgaggtgtggaaacctttccctcagag
caccgtcagatttcctggcgatgtttataaggtgtataggtacaatgcagttttagatcctctaattactgcgttgctggggactttcg
atactaggaatagaataatcgaagtagagaaccagcagagtccgacaacagctgaaacgttagatgctacccgcagggtagac
gacgctacggttgcaattcggtctgctataaataatttagttaatgaactagtaagaggtactggactgtacaatcagaatacttttga
aagtatgtctgggttggtctggacctctgcacctgcatcttaa

Primer Sequence Design of ToMV isolates in the Microbes Project

Designed Forward Sequence : TCCGGAAACTCACAACCCTTTG

Designed Reverse Sequence: GCTGCATGTTTGGCTTCGAT

Designed Probe Sequence: AAGTACAGCAGACTGTCGTCACCGC

REAL-TIME: Probe Hybridization

The SensiFAST™ Probe Hi-ROX Kit (BIOLINE) was used to validate the sensitivity and specificity of detection of ToMV RNA. The kit was specifically formulated for use with probe-detection technology, including Di-Hybrid BHQ Probes (Figures 10 and 11)

Reaction mix composition: Prepare a PCR mastermix. The volumes given are based on a standard 20 µl final reaction mix and can be scaled accordingly.

Milestone 2: Characterization of fungal and viral proteins. This milestone will achieve the goal of performing proteomic analysis of *R. solani* and tomato mosaic virus proteins with the aim of identifying protein signatures that can be used to discriminate between the viral and fungal models.

Task 2: Characterization of Plan viral proteins in Plant Model system
One of the most important steps in the microbial forensics project is the identification of distinguishing and targetable signatures/patterns in the model organisms. This milestone had two specific Aims, including 1) discovery of distinguishing and targetable protein signatures/patterns in the model viral organisms using 2D-DIGE and 2) isolation and identification of proteins from the model organisms using nano-LC/MS/MS.

A total of two specimens were analyzed for proteomics analysis with the aim of identifying proteins that show differential expression.

Code	**Sample**
OHKS	infected tobacco leaf
OGK	healthy tobacco leaf

To archive this milestone we used a combination of one dimensional polyacrylamide gel electrophoresis (1D PAGE) and two dimensional difference in-gel electrophoresis (2D-DIGE) technologies to screen the model organisms. The 1D PAGE allowed us to determine the generalized pattern of protein distribution and relative mass range of distinguishing protein bands. Based on the 1D-PAGE data we were able to design 2D-DIGE experiments that allowed us to profile and measure protein expression differences between plants that are infected by the virus and those that were not infected. A representative 1D gel image showing the electrophoretic pattern of the fungal purified virus and purified virus protein (Figure 1A) and fungal proteins (Figure 1B) are presented. This very important step is prerequisite to designing methods and devices that will target selected viral proteins.

The central goal of the 2D-DIGE experiment was to identify proteins that show qualitative and quantitative differences in the model organisms. This step is prerequisite

to determining which proteins are present at different levels, and which are present in one organism and not in the second. We compared the protein expression in a tobacco plant infected with the virus and a plant that is not infected with the virus.
This experiment was designed to allow us test the sensitivity of our assay systems in detecting the presence of a virus in a plant. The ability to detect virus proteins (a hypothetical threat agent) in plant tissue will suggest that our system will be capable of detecting a real viral threat agent that is in a plant or benign fungi like the bread mold. As shown in Figure 2, the 2D-DIGE technology was effective in detecting differences between infected and non-infected leaves.

- The samples were further cleaned up by precipitating with ToPREP and re-suspending in fresh ToPI-DIGE protein isolation buffer to remove any interfering substances that may affect the first dimension IEF.

- Protein assay was performed on all samples using ToPA to determine the protein concentration.

- A volume that equaled 50ug of protein from each sample was aliquoted in a tube and labeled with 200pmoles of either cy3 or cy5 protein labeling dye using GE Healthcare Bio-Sciences protocol.

- A standard pool of all samples was prepared by combining equal aliquots of protein from each sample and labeled with 200pmoles/50ug protein of cy2 protein labeling dye using the standard protocol.

- The labeled samples were matched so that one cy5 labeled sample and one cy3 labeled sample were mixed together. To this mix 50ug of the labeled standard pool was also added.

- The mixed samples were loaded on a 24cm, pH 3-10 IPG strip by rehydrating the dry strip with 450ul of rehydration buffer that contained the samples. The strip was rehydrated in this buffer for 24 hours, with 30volts applied to assist with the absorption of larger proteins.

- After rehydration the Strip was focused for a total of 65,000 volt hours.

- After the strips were focused to complete the first dimension they were processed by equilibrating them in SDS equilibration buffer and alkylated with iodoacetamide and reduced with dithiothreitol.
- The equilibrated strips were loaded on a 24cmx20cm 12.5% SDS-PAGE gel, and run to separate the proteins in the second dimension for 4.5 hours at 16watts per gel.

- The gels were then scanned on a GE Healthcare variable mode fluorescent scanner.The gels were scanned at 3 wavelengths for each cy dye on the gel.

- The images were cropped and loaded into Decyder software database for analysis.

- The images were first processed on decyder DIA module to detect protein spots on the gel.

- The images with the detected spots were then analyzed using the DeCyder BVA module. This module allows comparisons between gels using the standard pool cy2 labeled gel image. There was one sample per type so there was no statistics for this stage of the study.

Identification of the differentially expressed proteins:

This milestone is concerned with picking, in-gel digestion and sequencing of the target proteins. To archive this milestone we performed differential *in gel* analysis of 2D-DIGE gels toselect candidate spots that have unique patterns of expression.
We picked a total of 184 candidate proteins from the stained gels, performed robotic in-geldigestion and sequenced the proteins using nano-LC/MS/MS technology as described below.

In-Gel Digestion and LC/MS/MS.

184 protein spots that met the inclusion criteria were identified on the gel images, picked into96 well plates with the *Ettan* Spot Picker (GE Healthcare) and digested overnight at roomtemperature using the *Ettan* Spot Digester (GE Healthcare) as previously reported (**Somiari *etal*, 2003**). The plate containing the digests were evaporated to dryness and stored until massspectrometry to identify proteins by tandem LC/MS/MS.

To assign identities to the candidate proteins of interest, the tryptic digested samples were

reconstituted with ultra pure water and sequenced by tandem mass spectrometry as describedby us (**Boyiri *et al* 2009**). Specifically, nano-flow LC/MS/MS was carried out with nano-bore electrospray columns constructed from 360 mm o.d., 75 mm i.d. fused silica capillary with thecolumn tip tapered to a 15-mm opening. The columns were packed with 200 A 5 μm C18beads (Michrom BioResources, Auburn, CA) to a length of 10 cm. The mobile phase forgradient elution consisted of: a) 0.3% acetic acid, 99.7% water, and b) 0.3% acetic acid, 99.7%acetonitrile. The flow through the column was split pre-column to achieve a flow rate of 350nl/min.

All tandem mass spectra were acquired on a Thermo DECA XP Plus ion trap mass spectrometer (Thermo Corp., San Jose, CA) with the needle voltage set at 3 kV. Ion signalsabove a predetermined threshold automatically triggered the instrument to switch from MS to MS/MS mode for generating fragmentation spectra.

The obtained MS/MS spectra were searched against the NCBI non-redundant protein sequence database using the SEQUEST computer algorithm (**Yates *et al* 1995**).

Figure 3-5 show 2D-DIGE gels and spots patterns generated in DeCyder, and Table 2 and 3lists the proteins identified so far in the fungi and plant model systems.

MILESTONE 1

TASK 2: Characterization of fungal viral nucleic acids.

ii) Characterization of fungal *Rhizoctonia solani* viral nucleic acids.

a) Extraction of Total Double-stranded RNA and Gel Electrophoresis: Fungal cultures used in the study are listed in Appendix B Table 1. In all 12 cultures were tested based on culture characteristics including but not limited to phenotypic characteristics, growth patterns, number of nucleic heterokaryon (2 nuclei) or homokaryon (1 nucleus), and presence and absence of viral double-stranded (ds) RNA.

All such cultures were then cultivated in malt extract broth for 18-21 days as described by Bharathan and Tavantzis (1990) for subsequent dsRNA extractions and analysis. Total dsRNA were extracted from cultures grown concentrations in a liquid broth using the procedure of Morris and Dodds (1979) with few modifications as described by Bharathan *et al*., 2005. DsRNA eluted from CF-11 column were further treated with nucleases to remove DNA and single–stranded RNA contaminants. Each DNAse and RNAse digestion was followed by treatment with proteinase K for 30 min at 37 ^{0}C (Bharathan and Tavantzis, 1990).

Nuclease Digestion of Total dsRNA from select *R. solani* isolates

Part of the extracted dsRNA was treated with DNaseI followed by S1 nuclease (Fermentas) for 30 min each as recommended by the supplier. Aliquots (10 µl) of digested and non-digested dsRNA were separated on 1% Agarose gel containing 0.5 µg/ml ethidium bromide for 1 h at 120 V in 1x TAE buffer and then visualized under ultraviolet light.

Phenol Extraction Method of Total Nucleic Acids

About 100-400 mg mycelium was ground in liquid nitrogen using a mortar and pestle. To the ground mycelia, 0.5 ml of 1x STE buffer containing 1.5% SDS and 20 mg/ml Bentonite was added. The tubes were incubated at 60ºC for 10 min. One vol of Phenol : chloroform : isoamyl alcohol (25:24:1) was added and the tubes were incubated at RT for

20 min with shaking. The tubes were centrifuged for 10 min at 7,818 *x g*, and the supernatant was transferred to a new tube and re-extracted with Phenol: chloroform: isoamyl alcohol as described above. Nucleic acids were precipitated from the supernatant with 1 vol of isopropanol for 30 min at -70°C. The pellet was washed with 0.5 ml 70% ethanol, dried for 5 min at 50°C, and dissolved in 100-300 µl ddH2O.
The purified dsRNA samples were fractionated electrophoresis in 1% agarose gels in TAE buffer.

40 mM Tris, 2mM EDTA, 20 mM sodium acetate, pH 7.8) at 30 V/cm for 6-8 hours. Following electrophoresis, gels will be stained with ethidium bromide and nucleic acid bands will be visualized by UV trans-illumination and photographed using digital documentation system.

b) <u>Purification of viral particles of *R. solani* isolates</u>

Four isolates of *R. solani* 303, 357, 301, and 386 were grown in malt extract broth for minimum of 40 days, ~100 g of tissue was homogenized. 0.03 *M* Citrate PO_4 buffer was used for subsequent buffers containing (0.03 *M* $NaPO_4$ dibasic anhydrous, 0.03 *M* citric acid, anhydrous, pH 5.8). The homogenized tissue was subjected to extraction buffer (0.03 *M* citrate PO_4 buffer, 0.42% 0.5 *M* EDTA, 0.5% 2-mercaptoethanol, 0.1% Sodium Diethyldithiocarbamate trihydrate). This mixture was filtered using miracloth and filtrate was centrifuged at 11,000 g for 10 minutes at 4ºC using an Avanti Centrifuge J-25 Beckman Coulter low speed centrifuge. Supernatant was discarded and then high speed pelleted at 113,000 g for 90 minutes at 4ºC using a Bechman Coulter Optima L-90 K Ultracentrifuge. The pellet was then resuspended in pellet suspension buffer (0.03 *M* Citrate PO_4 buffer, 0.001 *M* dithiothreitol, 0.001 *M* EDTA). Once resuspended and rinsed thoroughly with pellet suspension buffer, sample was stirred slowly overnight at 2-8ºC. The solution was then high speed centrifuged through a sucrose cushion buffer (0.03 *M* Citrate PO_4 buffer, 25% sucrose and 0.001 *M* EDTA) for 4 hrs at 113,000 g. The supernatant was discarded and pellet was resuspended in a total of 4 mL of 0.03 *M* Citrate PO_4 buffer and allowed to stir slowly overnight at 2-8ºC.

c) Extraction of double-stranded (ds) RNA from partially purified virus particles

This solution was then NET treated, 2x NET buffer (0.2 *M* NaCl, 1.0 *M* Tris, 0.002 *M* EDTA) was added to equal volumes of purified virus and allowed to incubate at 65ºC for 10 minutes. Equal volume of phenol as purified sample was then added to the mixture. The solution was vortexed adequately and then centrifuged for 10 minutes at room temperature at high speed in a Biorad Model 16K Microcentrifuge. The aqueous phase was collected being careful not to touch the interphase and equal volumes of phenol and chloroform/isoamyl alcohol- 24:1 ratio, was added to the aqueous phase volume. The solution was centrifuged for 10 minutes at room temperature at high speed in a microcentrifuge. The aqueous phase was removed and added equal volume of chloroform/isoamyl alcohol to aqueous phase volume. The solution was centrifuged once more for 5 minutes at room temperature at high speed in a microcentrifuge. Aqueous phase was collected and 1/10 volume of sodium acetate was added to each sample and filled to the top of a microcentrifuge tube with cold absolute ethanol. These samples were then stored overnight at -20ºC.

Characterization of dsRNA from various Reduced Complement (RC) and *wild type* by Nucleic acid Hybridization.

The dsRNAs in gels from various RC isolates and wild type dsRNA were characterized by PCR cDNA cloning, PCR sequencing, REAL-Time PCR, by probe preparation and hybridization. Cloned dsRNA fragments (Bharathan et al., 2005) were also tested for their sensitivity and specificity using specific RNA probe. . The representative variants of the *wild* (heterokaryon) type fungal strains and several reduced (homokaryon) complement (RC) strains of *R. solani* were characterized and their potential targets identified at the molecular level for the presence of extra-chromosomal double-stranded (ds) RNA and proteins. Briefly, about 11 dsRNA belonging to 11 different *R. solani* isolates that included both *wild* type and RC strains were cloned and sequenced. A unique strategy was developed for amplification and cloning different middle (M)-size dsRNA fragments by synthesizing random primer-dN_6 (5'-CCTGAATTCGGATCCTCCNNNNNN-3'). Full-length PCR products representing several genomic segments were cloned into p-Drive cloning vector (Qiagen) and sequenced. The sequences were determined using Sanger sequencing with an ABI 3730

sequencer (Retrogen Inc. San Diego, CA). The sequences were compared for similarity against the non-abundant sequence databases of EMBL, EU, using the BLAST algorithm.

R-PCR Conventional PCR of purified dsRNA from *R. solani*

Prior to PCR, the DNA in RNase treated dsRNA was further removed by with gDNAse treatment (Qiagen). Reverse Transcription was done by adding Quantiscript Reverse Transcriptase, dN6 primers (Qiagen) with few modifications Appendix B Figure 1 and Figure 2

Because of the considerable variation in size, sequence heterogeneity, and complex structures associated with the nature of dsRNA several protocols were identified and modified or cDNA synthesis and cloning. The different manufacturers and types were used individually or in combination as indicated below. They included

i) Qiagen ii) BIO-RAD iii) Ambion

iv) Fermentas v) Combination of Ambion-BIO-RAD

Random PCR (rPCR)

Up to 100 ng of a mixture of the 5 dsRNA segments were mixed with 0.25, 0.5, 1 or 2 µM of the up-dN6 primer, incubated at 99ºC for 2 min and quenched on ice for 5 min. Two Units of RevertAid™ Reverse Transcriptase, 50 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 4 mM MgCl2, 10 mM DTT, 1 mM dNTPs and 20 U of RiboLock™ RNase Inhibitor were added, and the mixture was incubated at 43ºC for 1 h. At this stage, the cDNA was used either directly in the subsequent PCR or for the synthesis of second strand cDNA as follows. The cDNA was heated at 99°C for 2 min then quenched on ice for 5 min. Ten U of the Klenow Fragment, 50 mM Tris-HCl (pH 8.0 at 25°C), 5 mM MgCl2, 1 mM DTT, 0.5 mM dNTPs and ddH2O to a final volume of 50 µl were added. The reaction was incubated at 37ºC for 30 min. The dscDNA was purified with the NucleoSpin® Extract II, eluted in 30 µl ddH2O and stored at -20°C till use. To test the sensitivity of the rPCR, 10-8-10-1 µg were reverse transcribed in the presence of 2 µM of

the universal primer-dN6 and directly amplified as described above without the Klenow Fragment reaction.

PCR Amplification

Amplification of the dscDNA took place in a reaction mixture containing; 1 µl of cDNA, 1x Taq Buffer advanced, 1.5 U of Taq DNA Polymerase (5 PRIME), 2 mM MgCl2, 0.25 mM dNTPs and 1 µM of the UP primer. The thermal cycling was performed in a Biometra T1 thermo cycler as follows: one cycle at 94ºC for 2 min, 65°C for 1 min and 72°C for 1 min, then 35 cycles of 94ºC for 40 sec, 52ºC for 30 sec and 72ºC for 3 min followed by a final extension step at 72ºC for 8 min.

Single Primer Amplification Technique (SPAT)

Primer PC3 described by Potgieter *et al.* (2002) was ligated to the 3` ends of the dsRNA as follows. About 250 ng of PC3 primer were ligated to 200 ng of a mixture of dsRNAs at a molar ration of >40:1. The ligation mixture included: 50 mM HEPES/NaOH, pH 8.0, 20 mM MgCl2, 0.01% BSA, 1 mM ATP, 3 mM DTT, 10% (v/v) DMSO, 20% (w/v) (PEG)6000, 20 U of Ribolock Rnase inhibitor and 30 U of T4 RNA ligase in a final volume of 30 µl. The ligation components were incubated at 37°C for 6 h then at 18°C descending at a rate of 2°C per h down to 12°C. The dsRNA was purified with the NucleoSpin® Extract II kit, eluted in 40 µl ddH2O, and concentrated in the SpeedVac vacuum concentrator (Savant Instruments Inc.USA) for 10-15 min.

In another treatment, the PEG6000, DMSO, BSA, and Ribolock RNase inhibitor were omitted from the ligation mixture and the reaction was incubated overnight at 16°C. The primer-ligated dsRNA was purified from excess primer with the NucleoSpin® Extract II and used in the subsequent RT-PCR. 2. The reverse transcription, removal of the RNA and annealing of the cDNAs were carried out basically as described for the FLAC method below with one exception: that is about 100 ng of the Oligo (dT)18 were used to prime the PC3-dsRNA in the cDNA synthesis reaction.

Full length Amplification of cDNA (FLAC)

About 250 ng of PC3-T7 loop primer described by Potgieter *et al*., (2009) were ligated to 200 ng of a mixture of dsRNAs as described for the SPAT method above. The purified primer-ligated dsRNA was denatured at 98°C for 2 min in the presence of 1M betaine and 2.5% (v/v) DMSO then quenched on ice for 5 min. The cDNA synthesis reaction contained: 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 1 mM dNTPs, 20 U of Ribolock RNase inhibitor and 400 U of RevertAid™ Premium Reverse Transcriptase. The reaction was incubated at 50°C for 1 h followed by 15 min at 55°C. RNA was digested with 0.1 M NaOH at 70°C for 20 min, followed by the addition of 0.1M Tris-HCl pH 7.5 and 0.1 M HCl to neutralize the reaction. The cDNA was then incubated at 68°C for one h followed by 1-2 h at 65°C.

The amplification mixture, calculated for a final volume of 25 µl, contained: 5 µl of cDNA, 1x of the provided DNA polymerase buffer, 320 µM of each dNTP, 2 mM MgCl2 and 1.25 µM of PC2 primer and 2.5 U of one of the following DNA polymerases. Phusion® High-Fidelity DNA Polymerase with Phusion GC Buffer, Platinium DNA polymerase, Go Taq DNA polymerase with the colorless buffer, 5PRIME Taq polymerase with advanced buffer set, or Long PCR Enzyme Mix with the long PCR buffer. The mixtures were incubated in a Bio-RAD T professional thermo cycler at 72°C for 2 min followed by 95°C for 2 min and then subjected to 35 cycles of 95°C for 25 sec with an increment of 1 sec per cycle, 65°C for 30 sec and 68°C or 72°C (as recommended by the manufacturer) for 5 min followed by a final step of 72°C for 10 min.

Direct ligation of dsRNA into pJET1.2 and pGEM®-T vectors

About 200 ng of a mixture of the dsRNAs were ligated into the *E. coli* cloning vector pJET1.2 (Fermentas) or into pGEM®-T (Promega) at a molar ratio of about 4:1 (insert:vector). The ligation mixture contained 2.5 weiss U of T4 DNA ligase and 25 U of T4 RNA ligase, 1 mM ATP, 5% (w/v) PEG6000 and 40 mM Tris-HCl, 10 mM MgCl2, 10 mM DTT in a final volume of 15 µl. The reaction was incubated at 14ºC for 24 h. In another experiment the reaction was incubated at 14°C for 24 h then at 4°C for extra 24 h. Ligated plasmids were transformed into XL-1 blue *E. coli* competent cells by means of heat shock at 42°C for 1 min. The obtained clones were screened by means of either PCR

or restriction digestion. Moreover, part of the positive clones was sequenced. The experiment was repeated 3 times.

Cloning and Sequencing:

PCR products were purified from the agarose gel or directly from the PCR tube with the NucleoSpin® Extract II, cloned into pGEM®-T cloning vector or pJET1.2 (Figure 3) and transformed into *E. coli* competent cells either by heat shock or by electroporation as described in the following sections. The sequences were determined using Sanger sequencing with an ABI 3730 sequencer (Retrogen Inc. San Diego, CA). The sequences were compared for similarity against the non-abundant sequence databases of EMBL, EU, using the BLAST algorithm.

Preparation of electro-competent cells

About 1-2 ml of *E. coli* XL-1 blue cells were cultured in 500 ml LB medium under shaking at 37ºC until the optical density of the culture at wavelength of 600 measures 0.5- 0.6. The culture was incubated on ice for 20 min and then the bacterial cells were pelleted at 2,000 x g at 0-2°C for 15 min. The pellet was resuspended and washed two times with 250 ml and a third wash with 10 ml of ice-cooled ddH2O. Each of the washing steps was performed at 3,000 *x g* for 15 min. The pellet was resuspended in 800 µl of 7% DMSO, divided into 50 µl aliquots, frozen in liquid nitrogen, and stored at -80ºC

Preparation of Chemical Competent cells

E. coli NM522 was cultured on LB-agar overnight at 37°C. Several colonies were transferred into 1 l Erlenmeyer flask with 30 ml of SOB supplemented with 20 mM MgCl2 and cultured until the OD550 reaches ~0.5. The culture was transferred into a sterile glass-tube and incubated on ice for 15 min. The tube was centrifuged at 1500 *x g* at 4°C for 10 min, and then the pellet was resuspended in 10 ml TFB buffer, and incubated on ice for 10 min. The suspension was centrifuged as described above, and then the pellet was resuspended in 4 ml TFB buffer, and incubated on ice for 10 min. DND solution (140 µl) was added to the suspension, mixed gently, and incubated on ice for 15 min. The

last step was repeated once. The competent cells were transferred (200 µl aliquots) into Eppendorf tubes and used immediately for transformation.

Transformation of Competent cells

Chemical competent cells (50 µl) were mixed with the cloning vector an Eppendorf tube and heat-shocked at 42°C for 1 min in a water bath. In case of electrocompetent cells, the cloning vector was purified from salts after the ligation reaction by ethanol precipitation. About 20-30 µl of the competent cells were mixed with the purified vector and electroporated at 1250 V for 4-6 msec.
After the heat- or electric-shock, the tubes were incubated on ice for 2 min, then 700 µl of SOC medium were added, and the cells were cultured for 1 h at 37°C. The bacteria were cultured overnight on AIX-LB agar (100 – 150 µl/plate) at 37°C. White colonies were screened by PCR, using vector-based primers flanking the cloning site. Colonies with positive PCR results were cultured in LB-amp overnight at 37°C and plasmids were purified as described in the following section.

MiniPreps and Restriction Digestion

Plasmids were purified from transformed bacterial cultures using the Fermentas GeneJEt Plasmid Miniprep Kit. Restriction digestions with the appropriate enzymes were performed according to the manufacturer instructions.

RESULTS

a) **DsRNA profiling :Characterization of Total nucleic acid purified by CF-11 cellulose column**

Double-stranded RNA was consistently detected in all the isolates of the *R. solani* tested.

Two isolates of *R. solani* t that were homokaryon (single nuclei) TOM7 and RS 29.3 did not have any dsRNA associated and one isolate that was wild type heterokaryon (2 nuclei) also did not have any dsRNA (Table 2).

Agarose gel electrophoresis The sizes of the dsRNA were classified as Large (L) (>4.6 kilo-bases); Medium (1.1-4.5 kilo-bases); and Small (<1.0 kilo-bases) Figure 7 and Table

2. For the size distribution of dsRNA within each isolate of *R. solani* tested please refer to Figure 7.

The double-stranded RNA nature of the nucleic acid was established by nuclease treatment. All dsRNA fragments were resistant to DNAse and RNAse under high sal conditions (figure 6). Only nuclease treated highly gel purified dsRNA were further used for cloning and sequencing reactions of the project. Moreover, the output of our effort to profile viral dsRNA is shown in the Appendix B Figure 4 and Figure 5; dsRNA nature of viral nucleic acid in Figure 6; size distribution of different dsRNA in Appendix B Figure 7, Figure 8 and Table 2.

Only mid-size fragments of the dsRNA were further used for dsRNA characterization.

Results:

b) DsRNA profiling from Virus-like Particles (VlP's)

Wild type 303, RS357, and RS386 were all tested. Each of these isolates were found in the dsRNA from extraction procedure using CF-11 cellulose column chromatography were found to contain dsRNA. These isolates were subjected to the virus purification procedure as described elsewhere.

WT303, RS357, and RS386 were found to still contain mid size fragments which corresponded to the dsRNA isolated by the dsRNA extraction procedure Appendix B Figure 6 lanes 2, 3, 4 and 5. The band sizes of each of the tested isolates, WT303 (2.089 kb), RS357 (2.089 kb), and RS386 (1.962 kb) were found to correspond to the same number of base pairs seen when dsRNA was extracted by the CF-11 cellulose column.

It is consistent that fungal isolates that have viral infections have m-size dsRNA fragments that range between 1.1 and 4.5-kb.

c) Characterization of dsRNA from various Reduced Complement (RC) and *wild type* by PCR Cloning Nucleic acid Hybridization.

The representative variants of the *wild* (heterokaryon) type fungal strains and several reduced (homokaryon) complement (RC) strains of *R. solani* (Table 2) were

characterized and their potential targets identified at the molecular level for the presence of extra-chromosomal double-stranded (ds) RNA and proteins. Briefly, about 11 dsRNA belonging to 11 different *R. solani* isolates that included both *wild* type and RC strains were cloned and sequenced. A unique strategy was developed for amplification and cloning different middle (M)-size dsRNA fragments by synthesizing random primer-dN_6 (5'-CCTGAATTCGGATCCTCCNNNNNN-3'). The r-PCR products generated for representative dsRNA samples from the isolate 303 and 357 are shown in Figure 7. Full-length PCR products representing several genomic segments were cloned into p-Drive cloning vector (Qiagen) and sequenced. The sequences were determined using Sanger sequencing with an ABI 3730 sequencer (Retrogen Inc. San Diego, CA). The sequences were compared for similarity against the non-abundant sequence databases of EMBL, EU, using the BLAST algorithm. Several clones were picked; preliminary sequence comparison of some mycoviral dsRNA suggests 26-30% sequence identical to coat protein of Carrot Cryptic Virus, Beet Cryptic Virus, and the capsid protein of the White Clover Cryptic Virus. In order to test for sensitivity and specificity, probes were prepared from cloned dsRNA fragments of the wild-type dsRNA and hybridized to dsRNA and cloned DNA from both the other wild-type and RC strains *of R. solani*. Probes were dual labeled (5'FAM/3'BHQ-1) and the design for qPCR assays was done using Biosearch Technologies (Novato, CA) **Real Time Design™ software.** Thus, our discovery platform for the first time has developed a protocol to generate PCR products from unknown dsRNA sequences belonging to the genome of pleomorphic viruses infecting the *R. solani* isolates that are heterokaryon and a homokaryon.

Several *R. solani* isolates belonging to both the heterokarytic and reduced genome complements but containing M-sized dsRNA fragments were cloned. The conditions for rPCr and PCR were optimized depending on the isolates and the size of the dsRNA. For example One being dsRNA from 357 and the other was EGR 4. The isolate 357 is a heterokaryon with two nuclei and EGR-4 is a homokaryon with one nuclei generated from a basidiospore. The dsRNA nature of viral M-size fragment purified from 357 and EGR-4 was established by nuclease treatment (Figure 8 lanes 1 and Figure 9, lanes 1 and 2). Single size dsRNA of size 2.287 kilo base (kb) was consistently purified from 357, where as 3.301 kb dsRNA was detected in EGR-4 Figures 9 (b) and 8 (b), respectively.

All such highly purified nuclease treated dsRNA were subsequently used reverse transcription PCR and cloning. The dsRNA was resistant to DNAse and RNAse under high salt condition. The primer design and experimental protocol for optimum PCR amplification is shown in Figures 11 and 12. The optimum concentration was found to be 2.0 mM for 357 (data not shown), where as for EGR-4 it was 2.0 and 2.5mM (Figure 16, lanes 2 and 3, respectively. Little or no PCR products were detected at higher concentrations (Figure 16 lanes 4, 5, and 6).

We have for the first time shown the amplification of viral-dsRNA from *R. solani* that are both hetrokayon (reduced complement) and homokaryon (*wild type*). Thus, our discovery platform for the first time has developed a protocol to generate PCR products from unknown dsRNA sequences belonging to the genome of pleomorphic viruses infecting the *R. solani* isolates that are heterokaryon and a homokaryon.

The PCR products thus generated were cloned into pDrive Cloning vector (Figure 3) supplied in linear form with a U overhang at each end which ligates with high specificity to the PCR product. In all over 600 clones were generated for nearly all the 11 isolates of dsRNA viruses clone. All the white colonies that were selected for the isolates RS 113, RS 114, and T2 clones are shown in Figure 357 and 5 clones for the homokaryon EGR-4. The results from the enzyme digests of selected 7 and 5 clones from 357 and EGR-4 are shown in Figure 11, 12 , and 13 along with the electropherogram of the cloned digests. Several clones were selected that had varying sizes and were sequenced by (Retrogen, San Diego, CA, USA). The sequences were determined using Sanger sequencing with an ABI 3730 sequencer (Retrogen Inc. San Diego, CA). The sequences were compared for similarity against the non-abundant sequence databases of EMBL, EU, using the BLAST algorithm.

RESULTS:

Mini Preps and Restriction Digestion: In all plasmids were purified from over 600 clones and restricted enzyme digested. The results from select clones generated from PCR products from isolates 113, 114, T2, 303, 357, and EGR4 are shown in figures 11, 12, and 13 with inserts showing the electropherogram for each clone. The sizes were verified using the bioanalyzer data before sequencing. All such purified plasmids were

further tested for bioinformatic studies and experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis (milestone 2, task 2). They included:

i) Specificity of the primers

ii) Sensitivity of detection

iii) Accurate and statistically verified quantification of relative template abundance

iv) High PCR efficiency for more accurate quantification of the target nucleic acid using virus specific probe.

Primers and Probe Design from Cloned dsRNA Fragments: Based on the sequence data the Primers and probes were designed using the software on Bioline products for the isolates 303, 357, 386, EGR-4 and T2 Containing *R. solani* Virus Isolates

Direct Real-Time PCR Using Probes Designed to Detect Specific Viral dsRNA from Cloned rPCR Products

SensiFAST Probe One Step Kit (Bioline)

1. The primers and probes were diluted Dilute Primers and Probe to 1:10 from the stock (100uM).

2. A typical reaction mixture consisted of 2X SensiFAST Probe One-Step Mix, Forward Primer (10 uM), Reverse Primer (10 uM); Probe (10 uM); Reverse transcriptase; RiboSafe RNase Inhibitor; Template plasmid or dsRNA. All brought to a final volume of 50(µL).

3. The typical REAL-TIME PCR Running cycle was programmed for :

1 cycle	45 ^{o}C	10 minutes
1 cycle	95^{o}C	2 minutes
40 cycle	95^{o}C	5 seconds
	60^{o}C	60 seconds

SensiMix II Probe Protocol on Real-Time (Bioline)

1. The needed template mix (Plasmid DNA) was thawed, mixed and centrifuged for few seconds and kept on ice

2. The primers and probes were diluted to 1:20 from the stock (100uM)

3. A typical Reaction Mixture: A typical reaction mixture consisted of 2X Sensi Mix II Probe, Forward Primer (10 uM), Reverse Primer (10 uM); Probe (5 uM); Template plasmid. All brought to a final volume of 50(µL)
4. The typical REAL-TIME PCR Running cycle was programmed for :

1 cycle	95^{o}C	10 minutes
30 cycle	95^{o}C	10 seconds
	60^{o}C	60 seconds

RESULTS:

Validation of the Primer and Probe for target nucleic aid detection by REAL-TIME PCR Analysis

The specificity of the cloned fragments were tested by PCR amplification using specific primers and probe design for Real-Time PCR hybridization. Based on the sequencing data the forward and reverse primers were designed probes were synthesized and the type of probe and sequence is shown in Table 3.

Real-time PCR further confirmed these results (figure 14). The top panel for 357 suggests the Tm of the PCR product to be 86.9 ^{0}C with a cT value of 33. The primer sequence used was generated from the rPCR products of the virus isolate 357. The forward sequence and reverse sequence is shown in Table 3.

The results from EGR-4 suggests the TM of 87.4 with a cT value of 33. All such PCR products were at various times analyzed by gel electrophoresis confirming the PCR products data not shown. The primers designed from the cloned fragment of the dsRNA from 303, 357, and 386 showed a great degree of sequence relationship. However, there were high degree of specificity of the primers and probes for detection of the homologous dsRNA. These results were confirmed by the amplification efficiency.

Real-time PCR further confirmed these results Figure 14. The top panel for 357 suggests the Tm of the PCR product to be 86.9 ^{0}C with a cT value of 33. The results from EGR-4 (Figure 16) suggests the TM of 87.4 with a cT value of 33. The probe sequence comes fro the virus isolate 357 and the probe sequence is shown in Table 3.

In attempts to validate the specificity of the primers and probes from select isolates several experiments were designed to address amplification efficiency by establishing standard curve by performing series of dilutions of the template nucleic acids. These dilutions were then amplified with specific primers. Based on the analysis of the amplification profile position the efficacy of the approach is still limited by the necessary assumption that all samples are amplified with the same efficiency as predicted by the standard curve. So we took an approach for determining amplification efficiency by measuring the fluorescent readings from individual amplification process.

Various dilutions of the templates were done and amplified using the primers sequence from the isolate 357. The cT values ranged from 3 to 33 (Figure 17). In all about 57 clones were tested. The cT value of the primer alone was 29.33 (Figure 17). Similar results were obtained with primer 357 when tested with different target nucleic acid (Figure 18). The cT value of the primer alone was close to negative 29.33. This appeared to be the limit of detection.

Amplification Efficiency: I addition to the original data from the BIOFORCATION TAB, a condition was used for further analysis of the data. The condition states that only values of the samples with the melting curve as 1 shall be used for calculations. This indirectly states that any sample with melting curve of less than 1 has no significant relationship. With these calculations the results got even more significant. Similar computations were made which included Rn slope, amplification efficiency, and the intercept. These results are depicted in the figures 19 and 20.

The intercepts plotted for 303, 386, and 357 revealed the efficiency of 92%, 98.5%, and 98.3% , respectively (Figure 19).The intercepts plotted for EGR-4, 113, and 114 revealed the efficiency of 95.6, 85%, and 83%, respectively (Figure 20). These results suggest that there is strong relationship for comparison in these target nucleic acids.

To Test the Variations on Ct values with the increase in the Annealing Temperatures

Significance

- From the graph plotted Ct values vs Temperature, it is clear that Ct values are indirectly proportional to the increasing annealing temperature, but at a point i.e., 54°, the Ct value increases and stabilizes.

Significance

- From the graph plotted Ct values vs Temperature, it indicates that Ct values are indirectly proportional to the increasing annealing temperature, but at a point i.e., 54°, the Ct value increases and fluctuates in accordance to the given or tested temperature

Significance

- The plotted graph clearly indicates that the relation of Ct values with respect to the annealing temperature of various combinations of EGR4 has minimal fluctuations. There exists a stable relation between them.

To Test the Variations in Rn values with the increase in the Annealing Temperatur

- **Significance** As the graph indicates, there exists a lot of variations and fluctuations in theoutcome of their relation. However, at the temperature of 51° for sample 113.3, we can witness the most highest Rn value as compared to all the annealing temperatures. After the point of 57°, all the samples of 113 reveals a stable relation.
- **Significance** The sample 114, plotted against Rn values with that of the annealing temperatures reveals that at a point of 54° as the temperature, all the samples of 114 are at its peak However, after 57° as the increased temperature, there exists a stable relation amongst all.
- **Significance** The Samples of EGR4, indicates a minimal variations as compared to the rest of the sample (113, 114). All the samples reaches its peak at 54o as its temperature. After this point, the relation steeps down at 57o and exhibits a stable relation thereafter.

Validation of dSRNA probes prepared from the virus isolates 386, 357, 303. Probes to detect target nucleic acid molecules (viral dsRNA from) 386. The cTFAM ranged between 7 and 34. The negative target cT FAM was 34.18. The target nucleic with the highest dilution had a cTFAM value 22.11 (Figure 22 , 23, 24, and 25). These results suggest that the signature nucleic acid for detection target nucleic acid is specific and sensitive enough to detect very low concentration of the target nucleic acid.

Validation of dSRNA probes prepared from the virus isolate 357 to detect target nucleic acids from different isolates like 386, and 303 (figure 26). In all such study the probes from 357 were specific with itself (homologous) the CtFAM was between 7 and 17. It could also detect target nucleic acids from different other targets with similar specificity like in 386 target the CtFAM was as low as 3.83 and for the atrget nucleic acid from 303 it was as low as 7.33.

Milestone 2: Characterization of fungal and viral proteins. This milestone will achieve the goal of performing proteomic analysis of *R. solani* virus proteins with the aim of identifying protein signatures that can be used to discriminate between the viral and fungal models. This milestone is prerequisite for identification of proteins that will be eventually used to validate using the xMAP technology.

Task 2: Characterization of fungal and viral proteins for the development of a multiplexed assay

Experience teaches us that the use of multiple proteomic platforms with complementary strengths will improve the chances of realizing the specific aims of characterizing the proteome of the model fungi and viruses (Pastwa *et al 2007*). Thus, our proteomics analysis plan includes the use of 2D-DIGE, LC/MS/MS and xMAP technologies for discovery, identification and validation of the proteomic signature of the model organisms. As shown in Appendix C Table 1 different fungal samples were be analyzed using proteomics technologies.

The 2-D work-flow process for protein target identification is shown in Figure 1 Appendix C

Methods and Materials

Sample Preparation. 400 µL of lysate was precipitated using ToPrep Kit (ITSI-Biosciences, Johnstown, PA) using manufacturer's recommendation and the pellet was re-suspended in 2D gel electrophoresis compatible lysis buffer pH 8.5 (30 mM Tris-HCl, 2 M Thiourea, 7 M Urea, 4% (w/v) CHAPS and 1% NP40). Samples concentration was determined with ITSIPREP ToPA Kit (Bradford Assay, ITSI-Biosciences, Johnstown, PA).

Protein Labeling. Proteins (50 µg) were labeled with CyDyes DIGE Fluors (GE Healthcare, Bucks, UK) according to the manufacturer's recommended protocol (Cy3 or Cy5 fluorophores for samples, and Cy2 was used as the internal standard). Internal standard is a pooled sample of an equal amount of all samples in an experiment. Equal amounts of samples (50 µg each) were labeled with Cy5 and Cy3, respectively. The

internal standard labeled with Cy2 was added to the mixture and the volume was brought at 450 µL with 1X Rehydration Buffer (4% (w/v) CHAPS, 8 M Urea, 1 % (v/v) BioLyte Buffer (BioRad, CA, USA), and 13 mM DTT).

2D Gel Electrophoresis. The first dimension, isoelectric focusing (IEF) was performed in a 24-cm Immobiline DryStrip (BioRad, CA, USA) with a linear pH 3-10 gradient using Ettan IPGphor II (GE Healthcare), at 20 ˚C. The strips were loaded by rehydrating the strips for 12 hrs at 30 volts with final volume of 450 µL containing Rehydration Buffer and labeled sample. The focusing of proteins on the rehydrated IEF strips was performed in 5 steps: 1) 200 V for 1 hr, 2) 500 V for 1 hr, 3) 1000 V for 1 hr, 4) 3 hr gradient from 1000 to 8000 V, and 5) finally held at 8000 V until 65,000 total volt hours was accumulated. Prior to second dimension, the strips were equilibrated and reduced for 10 minutes in an Equilibration Buffer (50 mM TrisCl, pH 8.8, 6 M Urea, 30 % (v/v) Glycerol, 2 % (w/v) SDS) containing 0.5 % (w/v) DTT. The strips were reequilibrated and alkylated at room temperature on a shaker for 15 minutes in Equilibration Buffer containing 4.5 % (w/v) Iodoacetamide. The focused strips were loaded onto a 24 cm x 20 cm, 12.5% SDS-PAGE gel, and run for 4 hours at 16 Watts per gel.

Gel Image Analysis. The separated proteins labeled with Cy3 or Cy5 fluorophores, and Cy2 (the internal standard) were scanned and detected in gels using Typhoon Digital Imager with 3 different wavelengths. After detection, the images were analyzed using DeCyder analysis software (GE Healthcare), and candidate protein spots that were differentially expressed at a 2-fold or greater threshold were annotated. Differential Analysis Software (DIA) module in DeCyder software is used to perform protein spot detection and quantitation based on the area, slope, volume, and peak height of the proteins and the filtration is made to eliminate the spots that don't indicate the presence of a protein in a gel. The Biological Variation Analysis (BVA) module is used to perform gel to gel matching of spots, allowing quantitative comparison of protein expression across multiple gels which are matched to a Master gel (the gel with the most spots). Spots considered for picking were chosen based on a t-test of 0.05 or less and an average spot ratio of 2.0 or higher.

Protein Identification. The gels were stained with Sypro Ruby (Invitrogen, USA) and all protein spots that were processed using an Ettan Spot Handling Workstation (GE Healthcare). Picked gel plugs were destained in 50 % Methanol/20 mM Ammonium Bicarbonate. De-stained gel plus were reduced in 10 mM DTT / 20 mM ammonium bicarbonate for 15 minutes at 55 °C and then alkylated in 20 mM IAA / 20 mM Ammonium Bicarbonate for 30 minutes at room temperature. The dried plugs were rehydrated in 20 mM Ammonium Bicarbonate containing 1 ng/µL Trypsin Gold, Mass Spectrometry Grade (Promega, USA) and incubated overnight at 37 °C. After digestion, 50 % Acetonitrile/ 0.1 % Trifluoroacetic Acid was added to the plugs and incubated for 10 minutes at room temperature. The extraction solution was transferred to a clean tube and the above mixture was added again and incubated for 10 minutes. The two extraction solutions were combined, dried in the tube at 45° C for 2 hours and then analyzed by LC/MS/MS and database search.

Mass Spectrometry. The mass spectrometry used was a Thermo Finnigan LCQ Deca XP Plus operated in the positive ion mode with the spray voltage set at 1.8 kV. A Thermo Finnigan Surveyor MS Pump was used to deliver a linear acetonitrile gradient from 2 to 30% B over 30 minutes. Solvents consisted of water with 0.1% formic acid on channel A and acetonitrile with 0.1% formic acid on channel B. The MS Pump was connected to a PicoFrit column (New Objective) with a micro tee and the nanospray column was operated at a flow rate of 0.5 uL per minute. A "Top Three" method was a full MS scan from m/z 400-1500 was followed by MS/MS scans of the three most abundant ions to generate the experimental MS/MS spectra. Bioworks 3.3.1 SP1 software (Thermo Finnigan) was used to search the most recent NCBI non-redundant database with the following parameters: precursor mass tolerance of 1.4 Da, fragment ion tolerance of 1.0 Da, and trypsin was the selected enzyme. Proteins were identified when two or more unique peptides had X-correlation scores above 1.5, 2.0, and 2.5 for respective charge states of +1, +2, and +3 and the delta CN value was greater than 0.1.

DeCyder is an automated image analysis software suite which enables detection, quantitation, matching and analysis of Ettan DIGE system gels Differential Analysis

Software (DIA) and Biological Variation Analysis (BVA) are the two modules of the DeCyder software.

DIA is used to perform protein spot detection and quantitation. A volume threshold of 2.0 fold or higher is used to highlight the spots that are differentially expressed between compared samples. Based on the area, slope, volume, and peak height of the proteins a filtration is made to eliminate the spots that don't indicate the presence of a protein in a gel.

The BVA module is used to perform gel to gel matching of spots, allowing quantitative comparison of protein expression across multiple gels. For comparison purposes, an internal standard was used in the experimental design. This internal standard is a pooled sample of an equal amount of all samples in an experiment. The BVA module allows for the quantitative analysis across the multiple gels in the experiment. All gels in an experiment are matched to a Master gel (the gel with the most spots). Spots considered for picking were chosen based on a t-test of 0 0.05 or less and an average spot ratio of 2.0 or higher.

Mass Spec Analysis

The mass spec analysis were also done on the spots that were exclusive to Non-mutated parent only. The peptides for each protein is shown in the Table

These procedures included

Sample Cleanup:

•The gels were scanned on a Typhoon Digital Imager with 3 different wavelengths.

•The images were analyzed using DeCyder analysis software (GE Healthcare), and candidate protein spots that were present only non mutated parent were picked. These spots were annotated and analyzed using LTQ.

•Eluates were dried down using a speed vac and reconstituted in 2% Acetonitrile with 0.1% Formic acid.

•Samples were loaded onto a PicoFrit C18 nanospray column (New Objective) using a Thermo Scientific Surveyor Autosampler operated in the no waste injection mode.

Mass Spectrometry Conditions

•A linear Acetonitrile gradient was used to separate the tryptic peptides using the following conditions: at time 0 the Acetonitrile concentration was 2% and increased to 50% over 30 minutes with a flow rate of 300 nanoliters per minute at the tip and a 90% Acetonitrile wash was used at the end of the gradient for five minutes.

•A Thermo Scientific LTQ XL mass spectrometer was used for peptide sequencing using the following parameters: spray voltage was 1.8 kV, ion transfer capillary was set at 180 degrees Celsius, and a data-dependent Top 5 method was used where a full MS scan from m/z 400-1500 was followed by MS/MS scans on the five most abundant ions.

Methods for Identifying Proteins by Mass Spectrophotometry

Trypsin digested proteins are dried down in a vacuum concentrator and resuspended in 20uL of 2% Acetonitrile with 0.1% Formic acid. The peptide solutions are placed in glass autosampler vials and loaded into a Surveyor Autosampler Plus (Thermo Scientific)operated in the no-waste injection mode. After the sample loop is filled, the valve is switched into the HPLC flow path and the sample is swept into a PicoFrit column (New Objective, ProteoPep II C18, 0.075mm internal diameter X 100mm length) with a flow rate of 300 nanoliters per minute. A linear Acetonitrile gradient is used to separate the peptides based on their hydrophobicity using the following conditions:
at time 0, the Acetonitrile concentration is 2% and increased to 30% over 30 minutes. At 30.1 minutes the Acetonitrile concentration is increased to 90% and held for five minutes to wash the column. After the high organic wash, the pump is once again set to 2% Acetonitrile and allowed to run for 15 minutes for column re-equilibration. The peptides are eluted from the column into an LTQ XL mass spectrometer (Thermo Scientific). The spray voltage is set at 2.0kV and the ion transfer capillary is set to 180 degrees Celsius.

Database Search Parameters

Raw data files were searched against species-specific databases from NCBI using Proteome Discoverer 1.2 (Thermo Scientific) and the SEQUEST algorithm.

•Databases containing proteins from Genus Ustilago , Genus Fusarium, Genus Botrytis and Genus Rhizoctonia were searched using the following parameters: Trypsin as the selected enzyme with a maximum of two missed cleavages and a static modification of Carbamidomethyl Cysteine (+ 57.02 Daltons).

Proteins were identified when two or more unique peptides had X-correlation scores greater than 1.5, 2.0, and 2.5 for respective charge states of +1, +2, and +3 and the delta CN score was greater than 0.1.

Protein Target Identification: Here we used a combination of 2d-dige and nano-lc/ms/ms technologies to characterize the model organisms at the protein level. the work flow process is illustrated in Figure 1.

As shown in Figures 2, 3, 4, and 5 the 2D-DIGE system successfully revealed proteins characteristic of each model system. In fact several proteins were clearly detected in all isolates of *R. solani*. Models tested (Figure 2-5).

Protein Spot Analysis from Different Isolates of the *R. solani* Fungi: It is not unusual for host cell to respond differentially to viral infections. RS 29.7 does not have a virus and is a Reduced Complement isolate derived from parent isolate RS 29which is infected with the virus that has a M-sized dsRNA fragment. There are some proteins that are differentially expressed. Spot statistics data showed 2-fold increase or greater spot ratio threshold Figures 6 and 7). The mutant type RS29.5 had higher protein content that increased with no viral infections 114 sots (7.3%) compared to the wild type. Moreover when these spots were compared for their uniqueness—position on the gel, abundance, and spot volume ratio RS 29.5 had 88 and wild type parent isolate had only 10unique spots.

In some models the proteins were up regulated 29.3 A had two unique proteins Figure 7 left panel and in RS29 no unique proteins were found (Figure 8)

In fact some of the models had differential protein expression. Some of the proteins are up-regulated (Figure 9 and Table insert and Figure 10 Table insert). This relationship is significant since both the cultures are have reduced genome complement and are homokaryons. TOM7 isolate does not have any virus associated dsRNA and infect tomato plants where as T2 is infected with a Mid-size dsRNA and the fungus infects tobacco crop plants. It is not clear if these differences in the fungal genome content and the virus content contribute to virulence. However, definitely the difference in protein and the # of unique protein might have significant role in the infection process.

Our discovery platform was also able to detect proteins that show differential expression in the plant (Appendix A) and fungus in response to virus infection. DeCyder automated image analysis software suite which enables detection, quantitation, matching and analysis of Ettan DIGE system gels Differential Analysis Software (DIA) and Biological Variation Analysis (BVA) are the two modules of the De Cyder software was used. DIA is used to perform protein spot detection and quantitation. A volume threshold of 2.0 fold or higher is used to highlight the spots that are differentially expressed between compared samples. Based on the area, slope, volume, and peak height of the proteins a filtration is made to eliminate the spots that don't indicate the presence of a protein in a gel.

The Biological Variation Analysis (BVA) module was used to perform gel to gel matching of spots thus allowing quantitative comparison of protein expression across multiple gels. For comparison purposes, an internal standard was used in the experimental design. This internal standard is a pooled sample of an equal amount of all samples in an experiment. All gels in an experiment are matched to a Master gel (the gel with the most spots). Spots considered for picking were chosen based on a t-test of 0 0.05 or less and an average spot ratio of 2.0 or higher. Graph view 29-3.A vs. TOM7- up regulated in 29-3.A. Spot number 1988; T-test 0.014 Volume ratio 30.33 (Figure 12). The Graph view of 29-3.A vs. TOM7- down regulated in 29-3.A . The Spot number

is 1643 and the T-test 0.030 Volume ratio -18.42 (Figure 13). The Graph view 29-3.A vs. TOM7- down regulated in 29-3.A; Spot number 1643 T-test 0.030 Volume ratio -18.42. The graphical view of the standardized log abundance of proteins are shown in Figures 26 and 27. This is based on spot volume calculated by the DIA module. The spot abundance between gels is standardized using the internal standard. The dots on the graph are the actual standardized log abundance of the spot on a gel. The blue line shows the trend between the spot on different sample types (Tom7, 29-3A, T2). It also shows the point of the average abundance between the spots. The black lines connect the spots that are on the same gel. For example the top abundance spot on Tom7 is on the same gel as the lower abundance spot on T2 (Figure 24).

Two protein spots were picked from the fungal and identified by LC/MS/MS using the parameters described in the materials and methods section. Our focus was to profile the plant and fungal model systems at the global level to identify proteins that are characteristic of each model. Secondary focus was; a) to determine if the presence of viruses can be detected in the plant and fungal models and b) if the presence of the virus will cause differential protein expression. Preliminary assessment of the identified proteins indicate that some are unique to each model, and represent candidate targets that will allow the selective detection of the target with high sensitivity and specificity. The sequencing of the genome of *R. solani* will achieve the goal of elucidating the genome of these *R. solani* variants and developing a peptide database that

Graph view 29-3.A vs. TOM7- down regulated in 29-3.A

Spot number 1643

T-test 0.030

Volume ratio -18.42

Shows the graphical view of the standardized log abundance. This is based on spot volume calculated by the DIA module. The spot abundance between gels is standardized using the internal standard.

The dots on the graph are the actual standardized log abundance of the spot on a gel.

The blue line shows the trend between the spot on different sample types (Tom7, 29-3A, T2). It also shows the point of the average abundance between the spots.

The black lines connect the spots that are on the same gel. For example the top abundance spot on Tom7 is on the same gel as the lower abundance spot on T2.

Graph view 29-3.A vs. TOM7- up regulated in 29-3.A

Spot number 1988

T-test 0.014

Volume ratio 30.33

Graph view T2 vs. TOM7- down regulated in T2

Spot number 2131

T-test 0.029

Volume ratio -3.41

Graph view T2 vs. TOM7- up regulated in T2

Spot number 1048

T-test 0.038

Volume ratio 2.96

Graph view T2 vs. 29-3.A- down regulated in T2

Spot number 1585

T-test 0.017

Volume ratio -25.91

Graph view T2 vs. 29-3.A - up regulated in T2

Spot number 1609

T-test 0.038

Volume ratio 16.3

The Biological Variation Analysis (BVA) module was used to perform gel to gel matching of spots thus allowing quantitative comparison of protein expression across multiple gels. For comparison purposes, an internal standard was used in the experimental design. This internal standard is a pooled sample of an equal amount of all samples in an experiment. All gels in an experiment are matched to a Master gel (the gel with the most spots). Spots considered for picking were chosen based on a t-test of 0 0.05 or less and an average spot ratio of 2.0 or higher. Graph view 29-3.A vs. TOM7- up regulated in 29-3.A. Spot number 1988; T-test 0.014 Volume ratio 30.33 (Figure 12). The Graph view of 29-3.A vs. TOM7- down regulated in 29-3.A . The Spot number is 1643 and the T-test 0.030 Volume ratio -18.42 (Figure 13). The Graph view 29-3.A

vs. TOM7- down regulated in 29-3.A; Spot number 1643 T-test 0.030 Volume ratio -18.42. The graphical view of the standardized log abundance of proteins are shown in Figures 26 and 27. This is based on spot volume calculated by the DIA module. The spot abundance between gels is standardized using the internal standard. The dots on the graph are the actual standardized log abundance of the spot on a gel. The blue line shows the trend between the spot on different sample types (Tom7, 29-3A, T2). It also shows the point of the average abundance between the spots. The black lines connect the spots that are on the same gel. For example the top abundance spot on Tom7 is on the same gel as the lower abundance spot on T2 (Figure 24).

DIA is used to perform protein spot detection and quantitation. A volume threshold of 2.0 fold or higher is used to highlight the spots that are differentially expressed between compared samples. Based on the area, slope, volume, and peak height of the proteins a filtration is made to eliminate the spots that don't indicate the presence of a protein in a gel.

The BVA module is used to perform gel to gel matching of spots, allowing quantitative comparison of protein expression across multiple gels. For comparison purposes, an internal standard was used in the experimental design. This internal standard is a pooled sample of an equal amount of all samples in an experiment. The BVA module allows for the quantitative analysis across the multiple gels in the experiment. All gels in an experiment are matched to a Master gel (the gel with the most spots). Spots considered for picking were chosen based on a t-test of 0 0.05 or less and an average spot ratio of 2.0 or higher.

Graph view 29-3.A vs. TOM7- down regulated in 29-3.A

Spot number 1643

T-test 0.030

Volume ratio -18.42

Shows the graphical view of the standardized log abundance. This is based on spot volume calculated by the DIA module. The spot abundance between gels is standardized using the internal standard.

The dots on the graph are the actual standardized log abundance of the spot on a gel.

The blue line shows the trend between the spot on different sample types (Tom7, 29-3A, T2). It also shows the point of the average abundance between the spots.

The black lines connect the spots that are on the same gel. For example the top abundance spot on Tom7 is on the same gel as the lower abundance spot on T2.

Graph view 29-3.A vs. TOM7- up regulated in 29-3.A

Spot number 1988

T-test 0.014

Volume ratio 30.33

Graph view T2 vs. TOM7- down regulated in T2

Spot number 2131

T-test 0.029

Volume ratio -3.41

Graph view T2 vs. TOM7- up regulated in T2

Spot number 1048

T-test 0.038

Volume ratio 2.96

Graph view T2 vs. 29-3.A- down regulated in T2

Spot number 1585

T-test 0.017

Volume ratio -25.91

Graph view T2 vs. 29-3.A - up regulated in T2

Spot number 1609

T-test 0.038

Volume ratio 16.39

Several protein spots were picked from the fungal and identified by LC/MS/MS using the parameters described in the materials and methods section. Our focus was to profile the plant and fungal model systems at the global level to identify proteins that are characteristic of each model. Secondary focus was; a) to determine if the presence of viruses can be detected in the plant and fungal models and b) if the presence of the virus will cause differential protein expression. Our assessment of the identified proteins indicate that some are unique to each model, and represent candidate targets that will allow the selective detection of the target with high sensitivity and specificity.

CONCLUSIONS: We have successfully identified candidate RNA and dsRNA targets protein targets in fungal and plant model systems using the nucleic acid and 2D-DIGE and LC/MS/MS technologies. The next phase of the project will involve identification of more of the proteins that we picked from the gels, full characterization and validation of

identified proteins of interest. Ultimately, we will assign the proteins to biochemical pathways and evaluate their suitability as protein targets.

The model agent in addition to harboring the virus-like particles is also a important fungal pathogen causing very important diseases in cultivated crop plants (Figure 16). It causes blight, damping off and they are known to produce toxin during infection process. *F. graminearum* (teleomorph *Gibberella zeae*) is the causal agent of *Fusarium* head blight in wheat, barley, and oats and *Gibberella* ear rot in maize in temperate climates worldwide. It synthesizes trichothecene mycotoxins during plant host attack to facilitate spread within the host. In order to study proteins and pathways that are important for successful host invasion. In vitro conducted experiments in which *F. graminearum* cells were grown in aseptic liquid culture conditions conducive to trichothecene and butenolide production in the absence of host plant tissue. Protein samples were extracted from three biological replicates of a time course study and subjected to iTRAQ (isobaric tags for relative and absolute quantification) analysis.

Statistical analysis of a filtered dataset of over 230 proteins in the present study (Figure 12, 13, and 14) exhibited significant changes in expression, 72 of which were un-accumulated relative to their level at the initial phase of the time course. There was good agreement between up accumulated proteins identified by 2-DE-MS/MS and iTRAQ. RT-PCR and northern hybridization confirmed that genes encoding proteins that were up-regulated based on iTRAQ were also transcriptionally active under mycotoxin-producing conditions.

Numerous candidate pathogenicity proteins were identified using this technique, including many predicted secreted proteins. Curiously, enzymes catalyzing reactions in the mevalonate pathway leading to trichothecene precursors were either not identified or only identified in one replicate, indicating that proteomics approaches cannot always probe biological characteristics. A total of 148 spots showing differences in abundance were identified. Among these spots, 33 spots were subjected to ESI-MS/MS, with 23 identified. Seven proteins including sporulation-specific gene SPS2, triose phosphate isomerase, nucleoside diphosphate kinase, and woronin body major protein precursor were upaccumulated while 16, including enolase, saccharopine dehydrogenase,

flavohemoglobin, mannitol dehydrogenase, and malate dehydrogenase, were down accumulated. Variations in protein abundance were investigated at the mRNA level by real-time RT-PCR analysis, which confirmed the proteomic data for 9 out of the representative 11 selected proteins.

A data-dependent "Top Five" method is used for peptide sequencing where a full MS scan from m/z 400-1500 is followed by MS/MS scans on the five most abundant ions (Figure 16)

Database Searching:

Raw files from the mass spectrometer were searched against the following databases downloaded from NCBI: Genus Ustilago, Genus Fusarium, and Genus Botrytis using Proteome Discoverer 1.2 (Thermo Scientific)and the SEQUEST algorithm. Two very confident protein identifications were made and they included gi119364593 (Heat shock 70 kDa protein 2) with 8 peptide matches and gi71024269 (Actin) with 7 peptide matches.

To further validate the identifications, a program called Protein Prophet was used for database searching. For the Heat shock 70 protein, all of the same peptides were identified with at least 97% probability and all except for two had 99% probability. For the Actin protein, all of the same peptides were identified with 99% probability.

We have identified several proteins based on homology to the cousins of your Fungus (Fusarium and Ustilago) and I have put them in an Excel sheet (see attached). So far, the most confident ID came from the following protein with 8 peptides matching to the protein:

http://www.ncbi.nlm.nih.gov/protein/119364593

For some of the proteins we identify, they are also found in the SwissProt database which gives more information on protein function and other attributes, see the following link:

http://www.uniprot.org/uniprot/P18694

We have searched 200 data files so far and have many more proteins to put in the table.

DeCyder is an automated image analysis software suite which enables detection, quantitation, matching and analysis of Ettan DIGE system gels Differential Analysis Software (DIA) and Biological Variation Analysis (BVA) are the two modules of the DeCyder software.

Two very confident protein identifications were made and they included gi119364593 (Heat shock 70 kDa protein 2) with 8 peptide matches and gi71024269 (Actin) with 7 peptide matches. To further validate the identifications, a program called Protein Prophet was used for database searching. For the Heat shock 70 protein, all of the same peptides were identified with at least 97% probability and all except for two had 99% probability. For the Actin protein, all of the same peptides were identified with 99% probability.

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http://www.uniprot.org/uniprot/P18694

We have searched 200 data files so far and I have many more proteins to put in the table.

MILESTONE 3

TASK 1:

Develop and optimize protocol for Genomic DNA Extraction from Reduced Complement Isolates of *Rhizoctonia solani* 29.3A and T2 for Genomic Sequencing

This specific Aim was performed at Indiana University of Pennsylvania under the supervision of Dr N. Bharathan and Dr S. Bharathan. This was done for the development of a peptide database to allow a more efficient and accurate annotation of *R. solani* AG-1/AG-4 at the molecular level. The sequencing of the genome of *R. solani* will achieve the goal of elucidating the genome of these *R. solani* variants and developing a peptide database that would allow a more precise annotation of *R.* ***solani*** **AG-1/AG-4 at the protein level**. Two haploid isolates of the culture that currently being sequenced are 29.3 A and T2.

High quality genomic DNA required sequencing from the selected isolates was developed and optimized (300 µg of genomic DNA in ~1 ml from each of the reduced complement strains with purity and A260 /A280 ratio of 2.0) as stipulated in the statement of work for the genomic sequencing (Dr. William C. Nierman, Ph.D. Professor Director, Infectious Diseases Program J. Craig Venter Institute wnierman@jcvi.org.

Extraction

1. Harvested 25g culture by using cool water, incubate culture in liquid nitrogen for about 10 minutes.
2. Use mortar pestle to grind culture into powder form, transfer a breaker or big bottle.
3. Add 100mL of CTAB extraction solution into a breaker.
4. Then add 50mL of CTAB/NaCl into it and wash the cylinder with 50mL of CTAB extraction solution.
5. Use the blender to blend for 2 minutes, by 30sec off and on.
6. Mix well and incubate for 40 minutes at 65^{o}C.

7. Add equal volume of Chloroform/Isoamyl, rock for 20 minutes.
8. Balance and centrifuge for 5minutes 7500xg at room temperature.
9. Recover the top (aqueous) phase, into a cylinder and add 1/10 volume of 65^{o}C CTAB/NaCl by using backside of the 10mL pipette.
10. Mix well and transfer it into new 30mL glass tube, balance and centrifuge it at 7500xg for 5 minutes in room temperature.
11. Recover the top layer into a new 30mL glass tube by using the backside of the 10mL pipette.
12. Add equal volume of cool -20^{o}C of isopropyl alcohol, cap and mix by inversion and keep at -20^{o}C for overnight.
13. Balance it with Isopropanol and centrifuge at 4000rpm for 5minutes in 4^{o}C (Avanti J-25 beckman).
14. Pour out the supernatant and invert the tubes for a minute.
15. Add 2mL of cool 70% EtOH to wash the pellet, balance and centrifuge at 4000rpm for 5 minute by 4^{o}C. Repeat this step by second time.
16. Pour out the 70% EtOH invert for a minute and keep on ice under the hood to let it dry. DON'T turn on UV light!!
17. Add 1mL of TE buffer to each tube and rock at Max Q 2000 until all dissolve.
18. Combine all the solution into one tube mix gently.
19. 1µL is used to screen at 260_A and 280_A by using Du 800 spectrophotometer for calculate the purity of the DNA by
20. 20 µL is used to run on 0.8% Agarose gel.

CsCl Centrifugation

1. Divide the total DNA volume into 2 and added each into a 15mL top tube.
2. 10.6g of CsCl are added into each tube, allow the CsCl to dissolve by the DNA sample.
3. TE buffer is added into each tube to bring the total volume into 10mL for help on dissolve the CsCl.
4. Slowly transfer all the solution into a polyallomer quick seal centrifuge tube.
5. 600µL of 10mg/ml of EtBr is carefully added to each of the tube.

6. Another 2mL of TE buffer is needed to fill the tube to the top and balance at the sametime.
7. The balanced tubes are centrifuge for 70K rpm at 20^{o}C for 40 hours by Optima L-90K Ultracentrifuge with rotor type 70.1 TI.

Draw the DNA band

1. Made a small hole on the top on the tube by using 18G1/2 needle and 5mL luer lock.
2. Draw the DNA band from the top on the band, into a 50mL top tube.
   *Do not do it from the bottom of the band, if so you may loss lot of the DNA.
3. Add isopropanol saturated into it to extract the EtBr out from the DNA.
4. Pippetted out the top layer of the isopropanol with EtBr into a solution of 5% Clorox.
5. Repeat step 3 and 4 until the DNA sample is completely clear.
6. Precipitate the DNA by adding:
   i) 10mL of TE buffer
   ii) 5mL of 7.5M ammonium acetate
   iii) Add 95% cold EtOH to top of the tube
   iv) Keep it at -20^{o}C for 1hour.
7. Balance the tube and centrifuge for 10K rpm for 10 minutes at 4^{o}C.
8. Pour out the 95% EtOH, invert for few minute and air dry it on ice.
9. Add 500µL of TE buffer to each tube, to dissolve the pellet.
10. Close it with parafilm and keep at 2^{o}C – 8^{o}C for overnight.

Re-precipitation of DNA

1. Add 1/10 volume fo 3M NaOAc, pH5.2 to the suspended solution to re-precipitate.
2. Add cold 95% EtOH to top up the tube and keep at -20^{o}C for overnight.
3. Centrifuge DNA sample at 14k rpm for 35 minutes at 4^{o}C.
4. Draw out the 95% EtOH and wash pellep with 500µL of 70% cold EtOH.
5. Centrifuge it at 10k rpm for 5 minutes at 4^{o}C.

6. Repeat for step 4 and 5 for 2nd time wash.
7. Airs dry the pellet on ice.
8. Re-suspend the pellet with 500µL of TE buffer, take 1µL to screen at 260_A and 280_A by using Du 800 spectrophotometer for calculate the concentration and purity of the DNA.
9. 300µL of the Genomic DNA is sending for sequencing and the left over is re-precipitated.

Buffer

1) 3M sodium acetate pH6
   a) Weight 20.40g of sodium acetate to 30mL of autoclaved water.
   b) Adjust the pH with glacial acetic acid to pH6.
   c) Add autoclaved water to the final volume of 50mL
2) Extraction buffer
   a) Mix 20mL of 1M Tris-HCl, pH8
   b) 20mL of 200mMEDTA,pH8
   c) 16.36g of NaCl
   d) 4g of CTAB
   e) 200mL of autoclaved water
   f) Incubate at 65°C of water bath until all dissolved
3) CTAB/NaCl solution
   a) 80mL of autoclaved water
   b) 4.1g of NaCl
   c) 10g of CTAB
   d) Final volume will be 100mL
   e) Incubate at 65°C of water bath
4) TE buffer
   a) Mix 150µL 200mM EDTA
   b) 300 µL 1M Tris-HCl
   c) 29.55mL autoclaved water

5) Isopropanol saturated
    a) Mix 15mL of 20XSSC
        i) Make 20XSSC by dissolve 175.3g of NaCl and 88.2g of sodium citrate into 800mL
        ii) Adjust the pH to 7.0 with a few drops of 1M HCl.
        iii) Add autoclaved water to the final volume of 1L.
        iv) Sterilize by autoclaving.
    b) 25mL of isopropanol
6) 7.5M ammonium acetate
    a) Dissolve 28.875g of ammonium acetate into 25mL of autoclaved water.
7) Keep enough of 95% ETOH and isopropanol at -20^{o}C.

The Results of purified genomic DNA and the subsequent PCR amplification using the following

(ITS1-F) CTTGGTCATTTAGAGGAAGTAA and

(ITS4-R)CAGGAGACTTGTACACGGTCCAG (Gardes & Bruns, 1993) reverse primers are shown in the Appendix D (Figure 1)

The annotation of R.solani strains T2 and 29.3 is being completed for data processing of the proteins Appendix D (Table 1 and Table 2)

MILESTONE 3

Task 2

The modern imaging methods into biodefense research will help elucidate mechanisms at the macro level that characterize host-microbe interactions. Our research at Indiana University of Pennsylvania funded by Defense Threat Reduction Agency (DTRA) in cooperation with ITSI-Biosciences and Department of Health uses fungal-viral model system to study microbial interactions; cytoplasmic exchange of genetic elements; protein factors; and the underlying causes of host cell death. In the present study using 19 different paired combinations (Appendix E Figure 5), we observed hyphal interactions between compatible and incompatible *Rhizoctonia solani* isolates using a compact image stacking device that allows one to photograph objects as small as 0.5 mm using a confocal camera. The staining technique for pair wide combination is shown in Appendix E, Figure 1. When the mycelia of *R. solani* encounter mycelia with a different genetic background, distinct barrage lines (BL) form (Figure 2, Figure 3, and Figure 5). Several incompatible reactions were observed (Figure 2) or compatible interactions were seen Figure 4 and Figure 6. Several ultra-high resolution images were generated showing high degree of stratification near or close to the tip of the fungal mycelium suggesting active protein and lipid synthesis. The device uses a a stacking device was developed earlier to capture multiple images in depth to compile in to a single fully focused image. The specimen mount itself can also move the specimen in the X and Y axes for shorter distances for stitching panoramic shots. The Z axis stack can be downloaded to a computer and stacked using any number of stacking algorithms. Edited images provided extra high resolution that allowed us to observe perfect hyphal anastomosis in compatible pairs (Figure 6). The hyphae never anastomosed with incompatible pairs (i.e., the hyphae remained parallel or crossed over without merging). These behaviors appear to result from the detection of one or more diffusible factors or due to up-take of viruses, toxins and traffic within fungal cells (figure 7). Our results also suggest that the attraction to other hyphae in pairs of incompatible isolates was increased by supplementation of the growing medium with activated charcoal. We will report data concerning programmed cell Death (PCD) with one or two approaching hyphae. The complex soil-borne fungus *Rhizoctonia solani* represent important plant pathogens that affect a wide host-range of vegetable and field crops, ornamentals and tree species worldwide causing consistent reductions in quality and economic yield. The combination of strains that differ in host range, pathogenicity, and culture characteristics makes the R.

solani complex a challenging task in terms of characterizing genetically distinct or similar fungal individuals. Since the first report of viral double-stranded RNA (dsRNA) genetic elements associated with R. solani, the etiology, epidemiology, and molecular biology of the disease have been studied extensively. The soil fungus *Rhizoctonia solani* is a potential threat to food biosecurity because it can kill or reduce the vigor of developing seedlings resulting in poor plant stand and productivity. The fungus is found in diverse soil habitats in temperate, neotropical, and tropical regions of the world and a pathogen of most cultivated and native species of plants, including various bioenergy crops such as conifers, maize, poplar, sorghum, soybean and switchgrass. The fungus also harbors double stranded RNA viruses that influence the parasitic and saprobic activity of the fungus and could be potentially exploited for agricultural-bioterrorism. However, our ability to detect and counter such threats is limited.

Results:

To our knowledge this represents the first demonstration of transmission of dsRNA between genetically different individuals of *R. solani* confirmed with imaging (figures 4 and Protein analysis Figures 8. We are confirming these results with nuclear and mitochondrial markers. All such isolates also show considerable variations in the protein content (figures 8). In fact in many of these cultures the recipient isolates showed considerable increase or decrease in protein content. These results suggest that transmission can occur between somatically incompatible solates but that maintenance of the dsRNA in the recipient isolates was not stable after repeated sub-culturing on nutrient medium. The dsRNA was maintained in isolates for 5 wk but was not detected thereafter. This data suggests that the dsRNA might not be stable when transmitted into a different cytoplasm in the new fungal host environment. Perhaps factors or cellular mechanisms other than somatic incompatibility present in the fungal host might interfere with the ability of the transmitted dsRNA to replicate and persist within the new host.

Appendix A

Milestone 1: Identification of Signature ssRNA nucleic acid in ToMV infected Plant Model

Table 1: Degenerate primers selected for the detection of ToMV RNA

Primer	Sequence	Corresponding amino acid sequence	Degeneracy/# of inosines	Assay/product size
TobRT up 1	5'-GARTAYSCIGCIYTICARAC-3'	EY)P/A)ALQT	32/3	ONE-STEP RT-PCR/568 bp
TobRT do2	5—BGCYTCRAARTTCCA-3'	WNFEAK	24/0	
TobN up3	5'-GGCGYTGCARACIATHGTITAYCA-3'	ALQTIVYH	24/2	Nested PCR/400 bp
TobN do4	5'-GTRTTICCIATRAAIGTIGTIACRTC-3'	DVTTFIGNT	8/5	

Note: B =C or G or T; R = A or G; Y = C or T; S = G or C; H = A or T or C; I = Inosine

The highly degenerate primers (Tobamo 1 and Tobamo 2) were designed from conserved motifs of the RdRp genes of tobamoviruses (Gibbs *et al.,* 1998). All primers were synthesized by Integrated DNA technologies and Invitrogen.

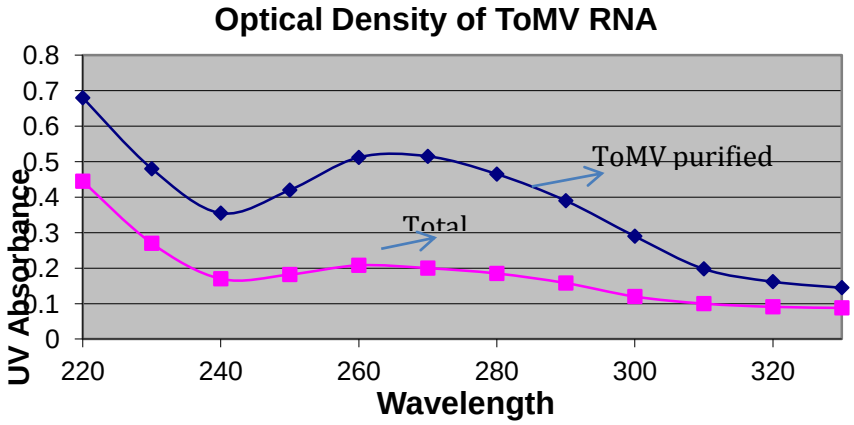


Figure 1 provides the absorbance spectrum for the partially purified ToMV ssRNA and RNA from infected leaf tissue. Both curves show a characteristic peak around 260 nm. The A260/A280 ratio, a measure of the purity of the nucleic acid with respect to protein contamination, was found to be 2.64. Using the A260 value, it was possible to obtain a

rough estimation of the concentration of nucleic acid in the sample, where purified virus ssRNA was ~8.8 µg/µL and the total RNA was ~0.6 µg/µL.

Electrophoretic Gel analysis of RNA (Appendix A Figure 2)

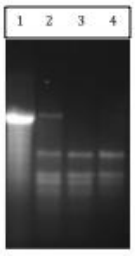


Figure 2: Lane 1: Purified viral RNA; Lane 2 Total Crude RNA from ToMV infected leaf ; Lanes 3 and 4 represent total RNA from non-inoculated Tobacco leaf.

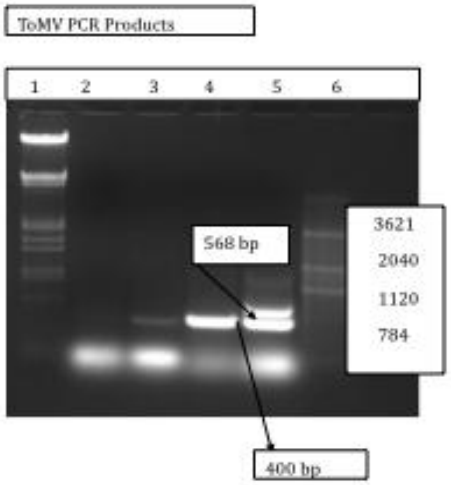


Figure 3. PCR products of various RNA samples are shown above. Lane 1 Marker, Lane 2, 3, 4, and 5 represent PCR products from the RNA from healthy leaf tissue, infected leaf tissue and purified ToMV viral ssRNA. Lane 6 contains pUC 19 Sau3 digested DNA. A conserved RdRp sequence was used in order to develop the appropriate primers. The PCR product is 570 bp in length.

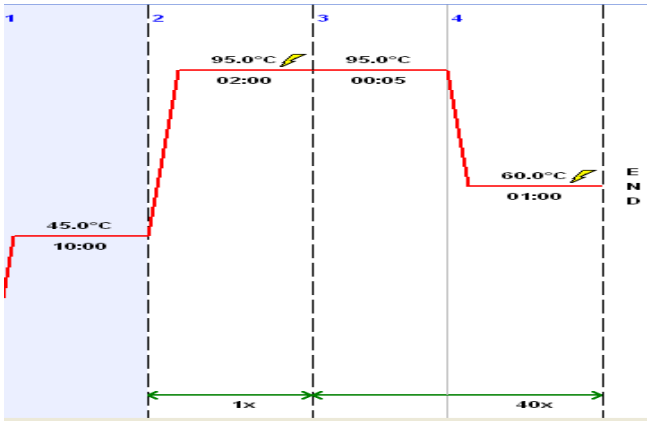
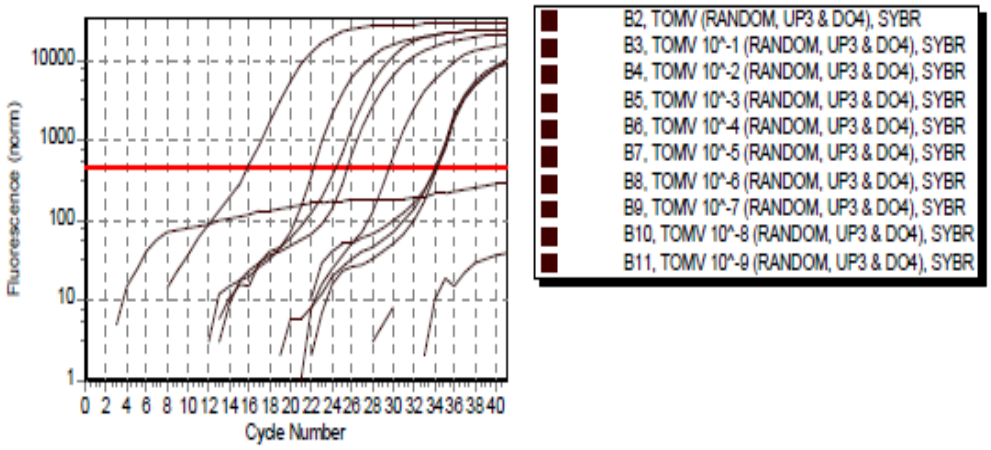


Figure 4: PCR Program:

Quantification SYBR

Pos	Name	Ct SYBR	Amount SYBR	Target SYBR
B2	TOMV (RANDOM, UP	34.16	-	
B3	TOMV 10^-1 (RAND	15.85	-	
B4	TOMV 10^-2 (RAND	24.36	-	
B5	TOMV 10^-3 (RAND	22.23	-	
B6	TOMV 10^-4 (RAND	29.61	-	
B7	TOMV 10^-5 (RAND	34.02	-	
B8	TOMV 10^-6 (RAND		-	
B9	TOMV 10^-7 (RAND	34.22	-	
B10	TOMV 10^-8 (RAND		-	
B11	TOMV 10^-9 (RAND	25.58	-	

Amplification Plot



Threshold 452 (Noiseband)
Baseline automatic, Drift correction OFF

Figure 5: PCR Amplification Profile with the cT values.

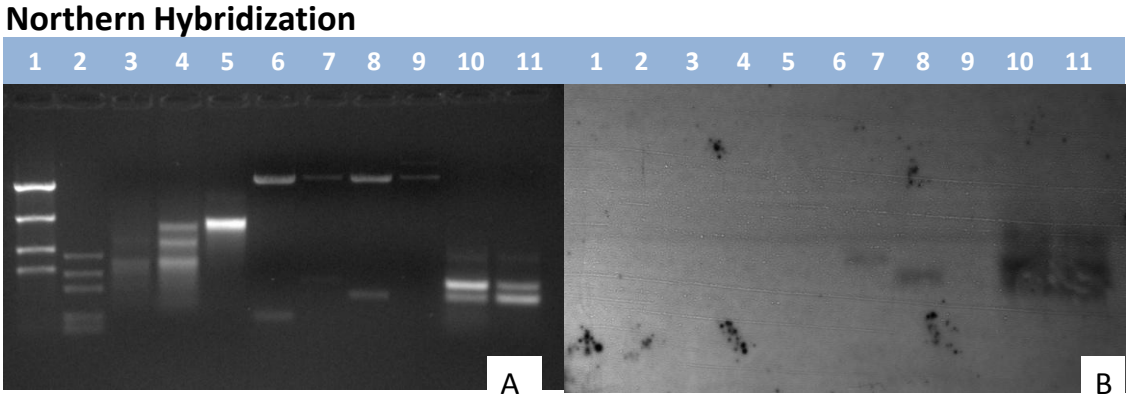


Figure 7 A and 7B

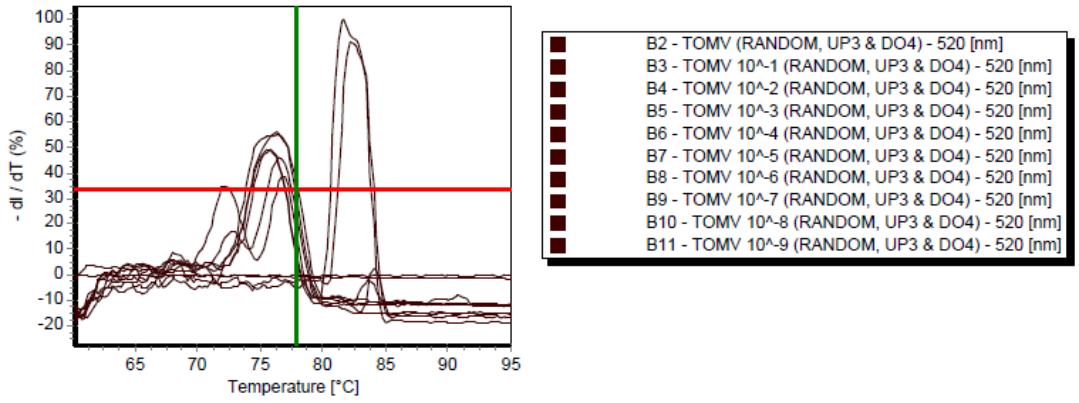
Ethidium Bromide stained gel of ToMV samples in preparation for Northern Hybridization. Lanes 1 and 2 contain IND-7 and PCR Markers , repectively. Lane 3 contains Total Healthy RNA, 4 contains Total Infected RNA, and 5 contains purified Viral RNA. Lanes 6, 7, 8, and 9 contains cut ToMV plasmids. Lanes 10 and 11 have samples of PCR products of Total Infected RNA and Viral RNA, respectively. Gel was run in order to transfer nucleic acid to nitrocellulose membrane for Northern Hybridization.

Northern Hybridization membrane exposed to film. PCR product of viral ToMV RNA used as probe for detection. Viral probe detected viral sequences in lanes 7, 8, 10, and 11. Samples in lanes 7 and 8 were ToMV plasmids cut with EcoRI restriction enzyme. Lane 10 contained PCR product of Total Infected RNA and lane 11 contained PCR product of PCR product of Viral RNA.

Melting Curve SYBR

Pos	Name	No. Tm SYBR	Tm x (°C) SYBR	Tm y (°C) SYBR
B2	TOMV (RANDOM, UP	1	75.8	
B3	TOMV 10^-1 (RAND	1	81.6	
B4	TOMV 10^-2 (RAND	1	82.3	
B5	TOMV 10^-3 (RAND	1	76.2	
B6	TOMV 10^-4 (RAND	1	75.6	
B7	TOMV 10^-5 (RAND	1	76.5	
B8	TOMV 10^-6 (RAND	0		
B9	TOMV 10^-7 (RAND	2	72.2	76.8
B10	TOMV 10^-8 (RAND	0		
B11	TOMV 10^-9 (RAND	1	76.4	

Melting curve



Threshold 33%

Figure 6: The stability of the PCR product was confirmed by Thermal melt analysis

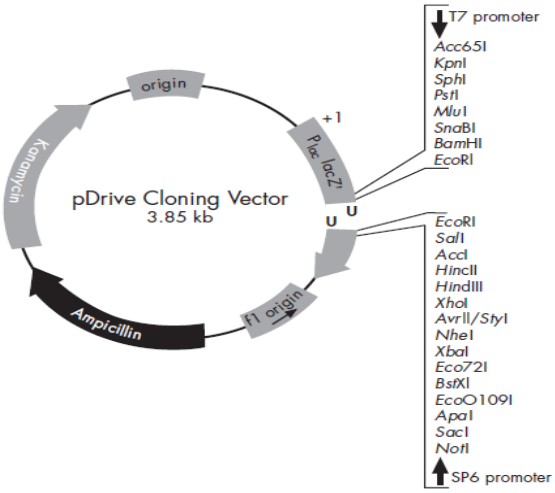


Figure 8: pDrive plasmid cloning vector with multiple cloning sites and reporter gene expression for clone selection.

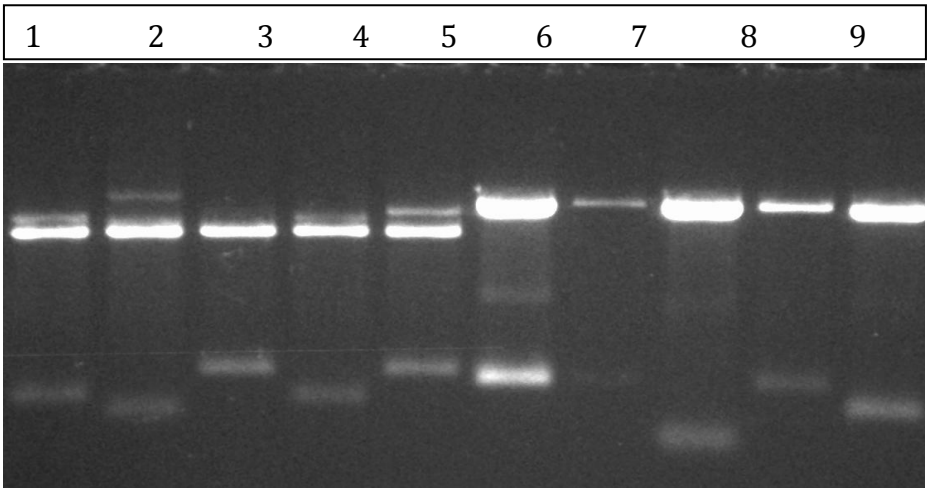


Figure 9: Gel Picture of ethidium stained plasmid DNA that were fast digest with EcORI DNA analysis of Cloned fragments of ToMV ssRNA following enzyme digests

Table 3: Sizes of the cloned fragments of ToMV from fast EcoRI digests

TOMV Plasmid	TOMV Plasmid	TOMV Plasmid	TOMV Plasmid	TOMV Plasmid	TOMV Plasmid	TOMV Plasmid	TOMV Plasmid	TOMV Plasmid
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9
MW (bp)	MW (bp)	MW (bp)	MW (bp)	MW (bp)	MW (bp)	MW (bp)	MW (bp)	MW (bp)
3436.1	4314.3	3289.8	3309.1	3653	3683.7	4022	3622.3	3837.5
3251.2	3270.5	877.9	1003.1	3289.8	1913.7	843.9	359.2	756.5
955.1	574.4		668.2	866.8	843.9			
679.2								

TOMV-Q-2-M13F & TOMV-Q-2-M13R

Align.	DB:ID	Source	Length	Score	Identities	Positives	E()
☑ 1	TR:B3VQW1_9VIRU	RNA polymerase (Fragment) OS=Tobacco mosaic virus PE=4 SV=1 *Cross-references and related information in:* ▶ Nucleotide Sequences ▶ Ontologies ▶ Protein Families	287	1293	98.4	99.0	6.9E-8
☐ 2	TR:B3VQW1_9VIRU	RNA polymerase (Fragment) OS=Tobacco mosaic virus PE=4 SV=1 *Cross-references and related information in:* ▶ Nucleotide Sequences ▶ Ontologies ▶ Protein Families	287	1293	98.4	99.0	6.9E-8
☑ 3	TR:Q88598_9VIRU	54 kDa protein OS=Tobacco mosaic virus PE=4 SV=1 *Cross-references and related information in:* ▶ Nucleotide Sequences ▶ Ontologies ▶ Protein Families ▶ Literature	474	1293	98.4	99.0	1.3E-8

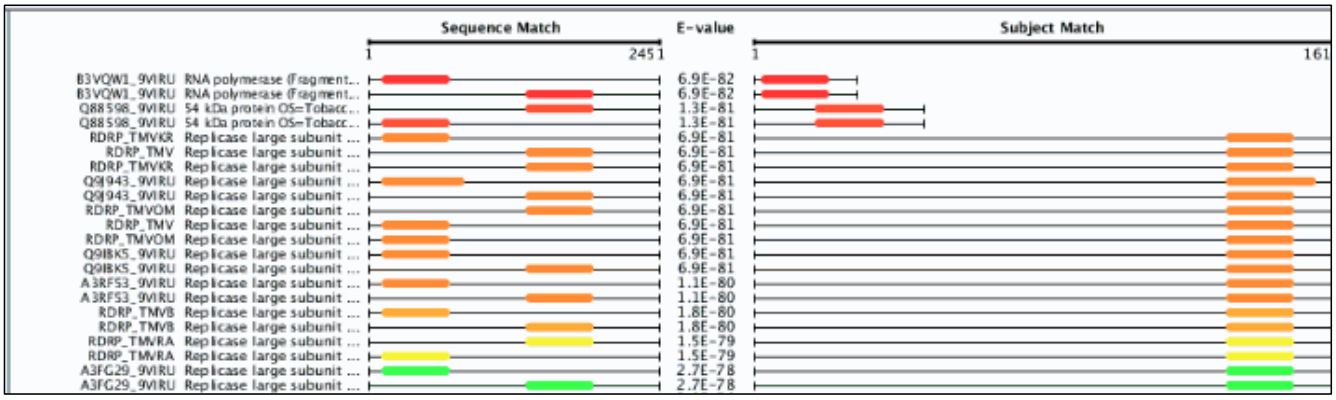


Figure 10. The sequence comparison to known Tobamovirus groups have shown 90% sequence

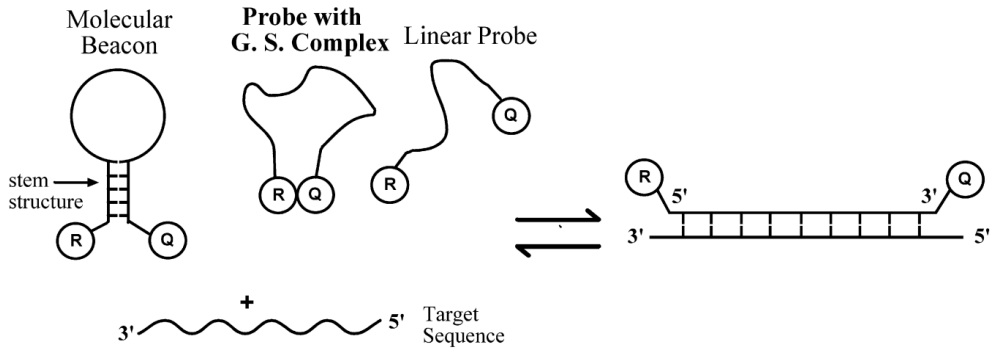


Figure 11: Dual Hybrid BHQ probes

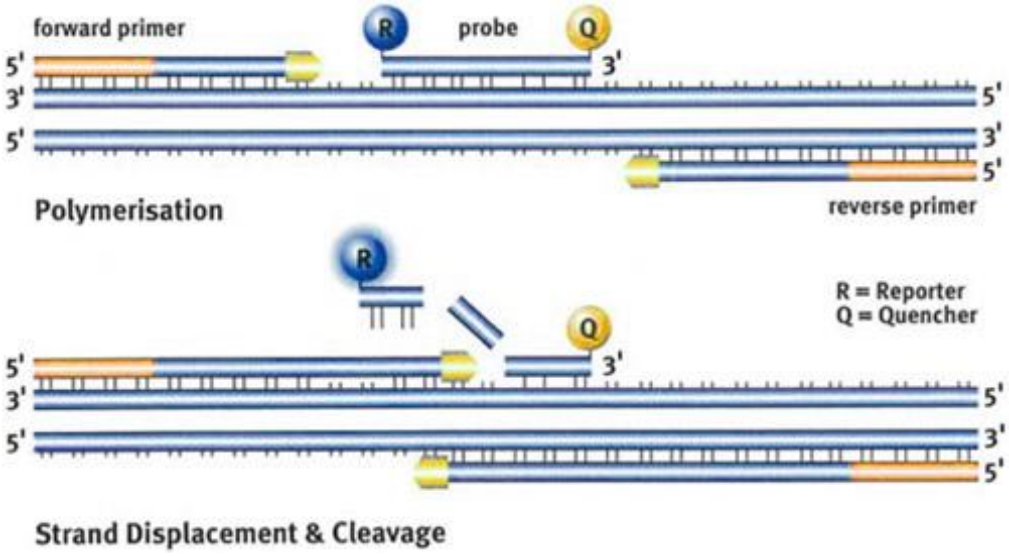


Figure 12: Principles of dual based probes

Table 4. SensiFast Probe Typical Reaction Mixer

Reagent	**Volume**	**Final concentration**
2x SensiFAST Probe Hi-ROX Mix	10 µl	1X
Forward primer	0.8 µl	400nM
Reverse primer	0.8 µl	400nM
10μMProbe	0.2 µl	100nM
Water	Up to 16 µl	
Template	4 µl	
	20µl Final volume	

Table 5. DNA Thermal Cycling Conditions

Cycles	Temperature	Time	Notes
1	95	5 minutes	Polymerase activation
40	95 60	10S 50S	Denaturation Annealing/extension (acquire at end of step

Table 6: Results of FAM data following hybridization using ToMV probe AAGTACAGCAGACTGTCGTCACCGC

.

eppendorf Eppendorf

Quantification FAM

Pos	Name	Ct FAM	Amount FAM
B2	357.3	5.60	-
B3	303.1	6.36	-
B4	RS113.1	23.78	-
B5	T2.1	3.71	-
B6	TOMV.2	25.11	-
C2	357.5	13.36	-
C3	303.2	5.49	-
C4	RS113.3	22.95	-
C5	T2.2	18.49	-
C6	TOMV.3	24.45	-
D2	357.7	7.04	-
D3	303.9	27.59	-
D4	RS113.5	22.70	-
D5	T2.3	19.04	-
D6	TOMV.4	26.63	-

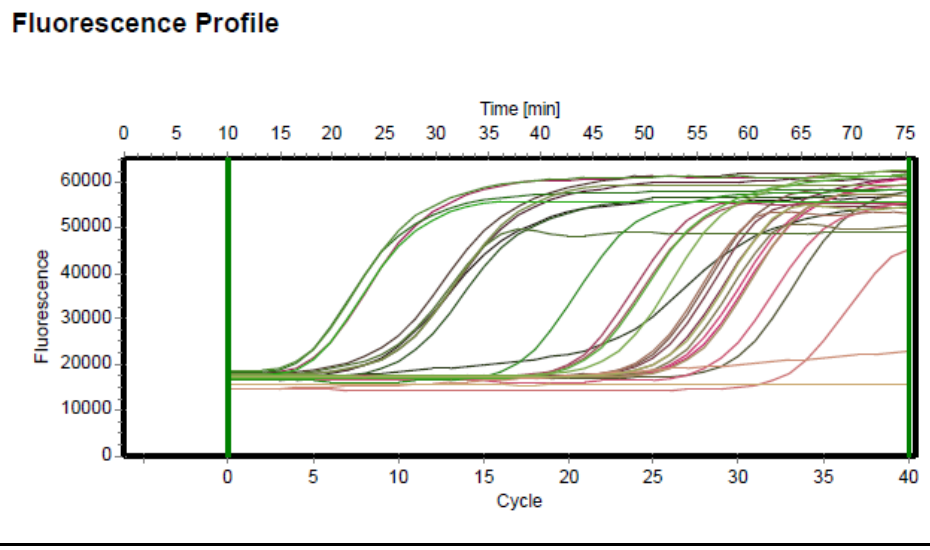


Figure 13: Fluorescent profile various concentrations of ToMV RNA hybridized to the probes as described in Materials and Methods.

Milestone 2: Signature Protein identification in ToMV infected Plant Model

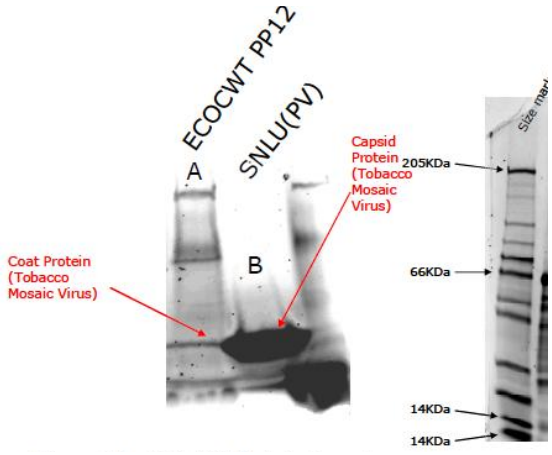


Figure 14: SDS-PAGE of purified proteins from ToMV infected and healthy leaf tissue. On the right is the electrophoretic banding pattern of BSA protein standards.

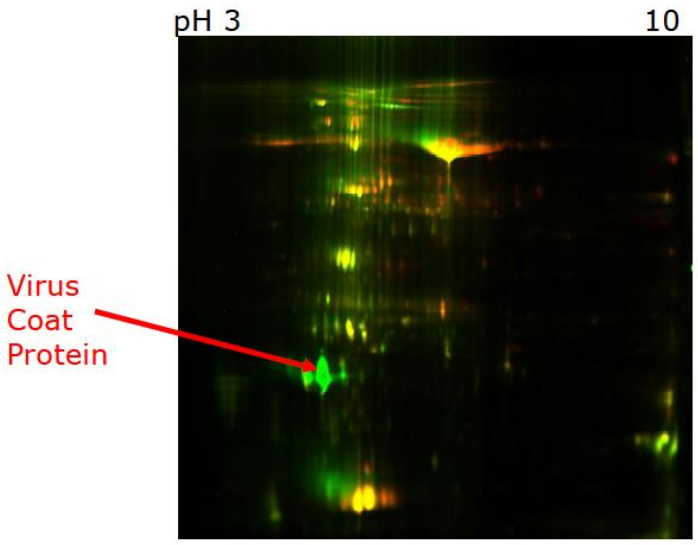


Figure 15: 2D-DIGE image showing the spot pattern and spot distribution of proteins isolated from ToMV infected and uninfected plants. Proteins colored green are more abundant in the virus infected plants and the proteins colored red are more abundant in the uninfected plants. The proteins colored Yellow are present at similar levels in both the samples. The virus coat protein are present only in virus infected plants.

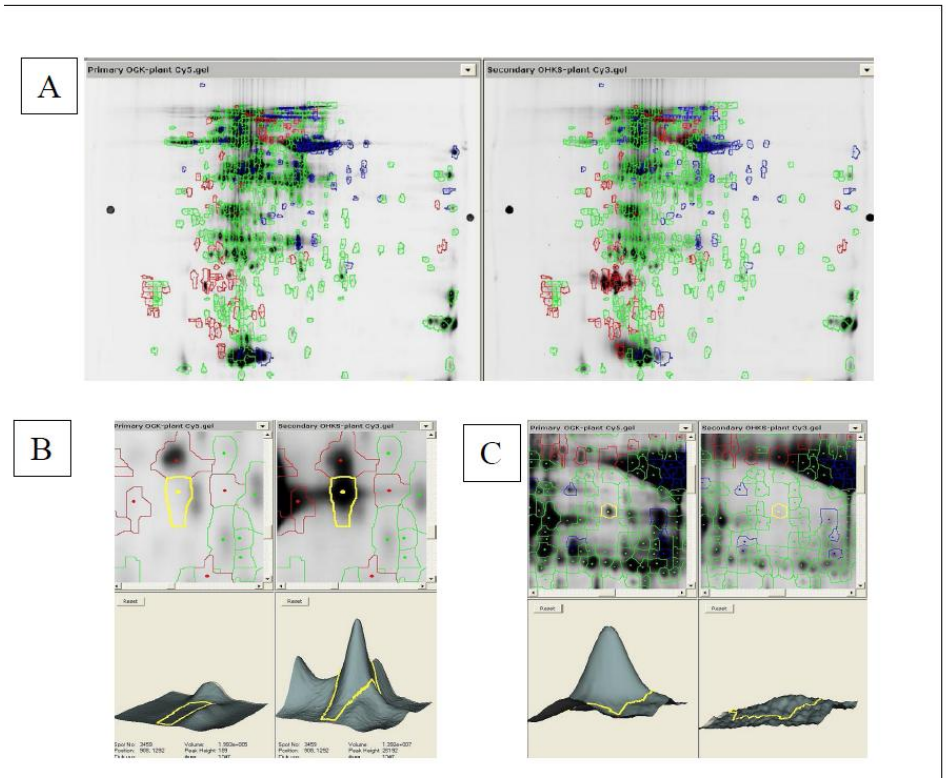


Figure 16: 2D-DIGE generated spot patterns from virus infected plants and uninfected plants (A). Global pattern of protein expression (B) a representative spot up-regulated in virus infected and (C) representative spot down-regulated in healthy plant

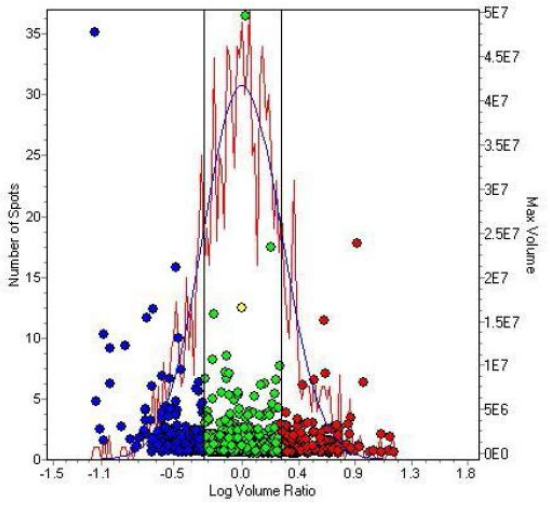


Figure 17: Over expressed (red) and under expressed (blue) protein spots in purified virus. The green spots occur at similar intervals in both samples.

Appendix B

Milestone 1: **Task 2** Characterization of fungal and viral nucleic acids. This milestone will achieve the goal of full characterization of viruses infecting *R. solani* and tomato mosaic virus RNA with the aim of identifying nucleic acid signatures that can be used to discriminate between the viral and fungal models.

Table 1: Isolates of R.solani that were tested for the presence and absence of Double-stranded RNA

Fungal Isolate Name	Growth Characteristics	Type of Nuclei	Genome Type
RS 303	Rapid Growth; Brown	Heterokaryon	Normal
RS 357	Rapid Growth; Brown	Heterokaryon	Normal
RS 386	Slower Growth; Brown	Heterokaryon	Normal
Rhs 113	Slower Growth; Brown	Heterokaryon	Normal
Rhs 114	Slower Growth; Brown	Heterokaryon	Normal
TE2-4	Rapid Growth; Downy	Heterokaryon	Normal
RS 29	Slower Growth; Pale yellow	Heterokaryon	Normal
RS29.3	Slower Growth; White and Downy	Homokaryon	Reduced Genome
T2	Slower Growth and white	Homokaryon	Reduced Genome
TOM-7	Rapid Growth and Brown	Homokaryon	Reduced Genome
EGR-4	Rapid Growth; white and Downy	Homokaryon	Reduced Genome
HUD 2-1	Brown and Rapid Growth	heterokaryon	Normal

Note: Heterokaryon isolates have 2 nuclei; whereas Homokaryon isolates have one nuclei

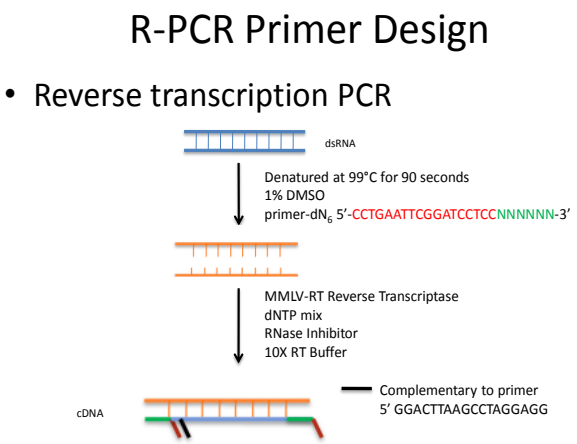


Figure 1: R-PCR Design of the random and reverse transcription

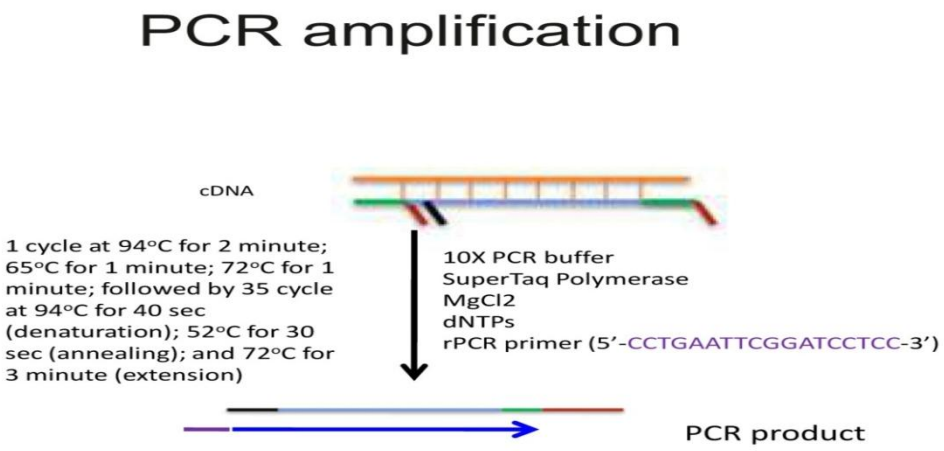


Figure 2: PCR Amplification Model

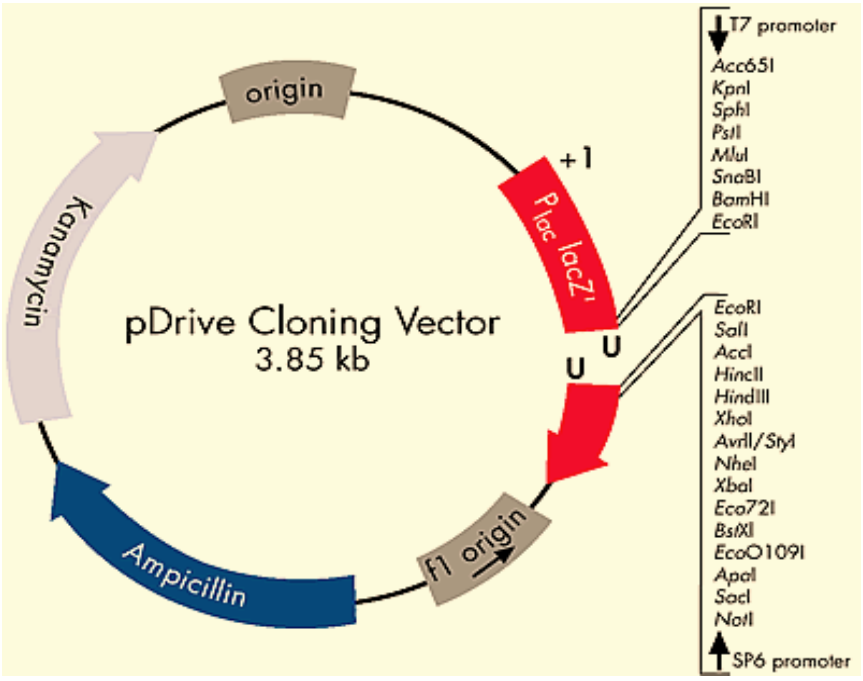


Figure 3. QIAGEN® pDrive cloning vector and the genes it expresses. ToMV was inserted in between the "sticky" U ends which then anneals together. *LacZ* flanks the insertion point which becomes expressed if target nucleic acid is not inserted .

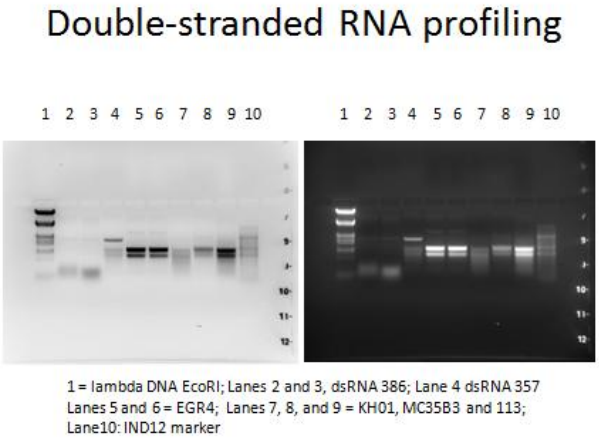


Figure 4: Ethidium bromide stained agarose gel showing the fractionation of dsRNA purified from *R. solani* isolates shown in Appendix B Table 1. The gels were stained with ethidium bromide and photographed using Kodak-pro Imaging System.

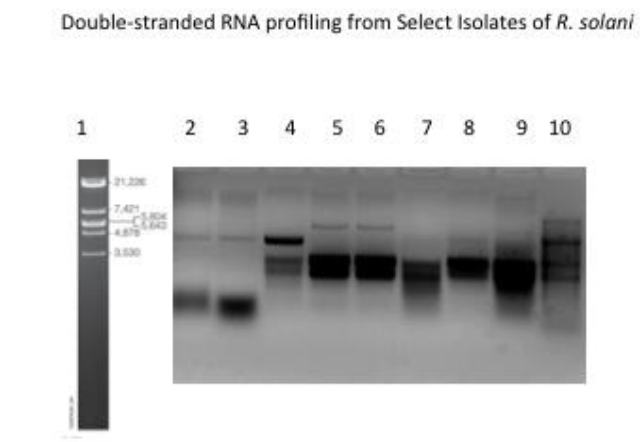


Figure 5: Double-stranded RNA was extracted from select isolates indicated above and analyzed by gel electrophoresis. The gels were stained with ethidium bromide and photographed using Kodak-pro Imaging System.

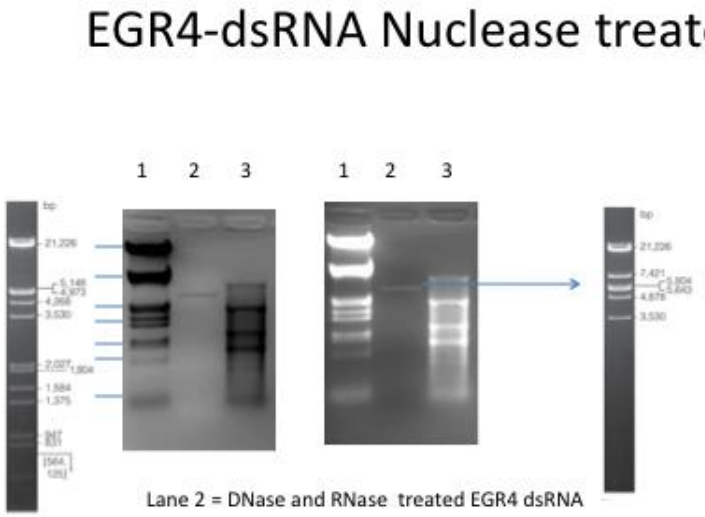


Figure 6: Nuclease treated dsRNA from the select isolate EGR-4 to establish ds-RNA nature of the viral nucleic acid. The gels were stained with ethidium bromide and photographed using Kodak-pro Imaging System.

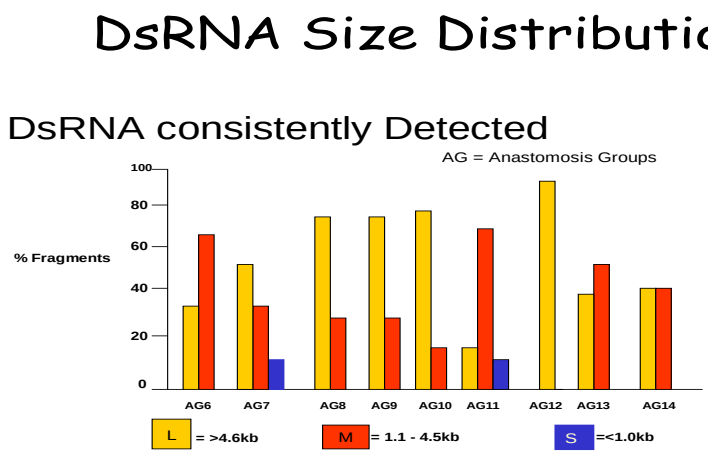
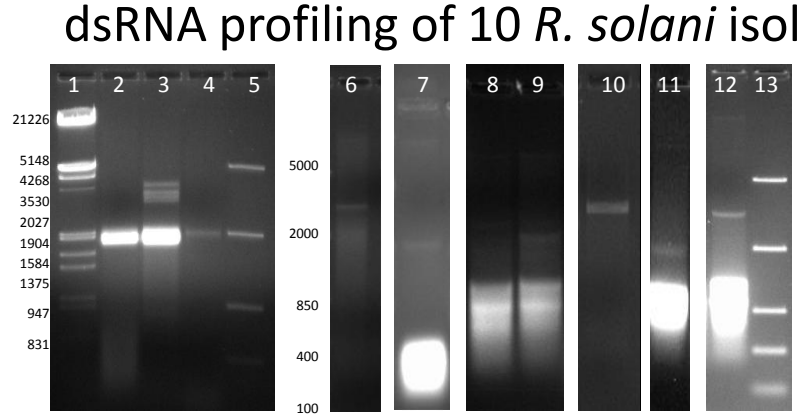


Figure 7: Size distribution profiling of dsRNA from viruses infecting different R. solani isolates. The dsRNA coild be broadly classified into Large (L) (>4.6 kilo-bases); Medium (1.1-4.5 kilo-bases); and Small (<1.0 kilo-bases).



Lanes	1	2	3	4	5	6	7
Sample	λ DNA HindIII/EcoRI	357	303	386	Middle Range Ladder	RS 29	T2
Sizes (bp)		1973.5	1983.4	2083		1291.4	2159.3
Lanes	8	9	10	12	11	13	
Sample	RS113	RS114	EGR 4	MC35B3	TE2-4	Middle Range Ladder	
Sizes (bp)	6650.7	6885.1	3489.3	2919.6	3500		
	2582.3	2601.8					
	1443.5	2108.6					

Figure 8: Size distribution profiling of dsRNA from viruses infecting 10 different R. solani isolates. The dsRNA coild be broadly classified into Large (L) (>4.6 kilo-bases); Medium (1.1-4.5 kilo-bases); and Small (<1.0 kilo-bases).

Table 2: Isolates of R.solani sizes of Double-stranded RNA

Fungal Isolate Name	Size of DsRNA	Type of Nuclei	Genome Type
RS 303	Medium	Heterokaryon	Normal
RS 357	Medium	Heterokaryon	Normal
RS 386	Medium	Heterokaryon	Normal
Rhs 113	Large, Medium , and Small	Heterokaryon	Normal
Rhs 114	Large, Medium, and Small	Heterokaryon	Normal
TE2-4	Medium and Small	Heterokaryon	Normal
RS 29	Mid-size Fragment	Heterokaryon	Normal
RS29.3	No dsRNA	Homokaryon	Reduced Genome
T2	Mid-size Fragment	Homokaryon	Reduced Genome
TOM-7	No dsRNA	Homokaryon	Reduced Genome
EGR-4	Mid-size Fragment	Homokaryon	Reduced Genome
HUD 2-1	No dsRNA	heterokaryon	Normal

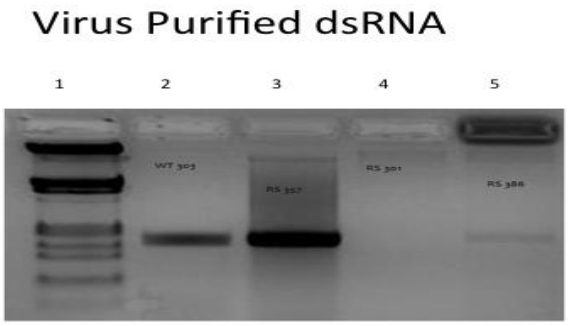
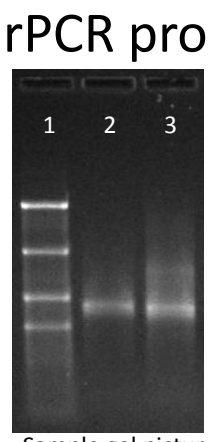


Figure 9: Viral Purified dsRNA from select isolates of *R. solani*

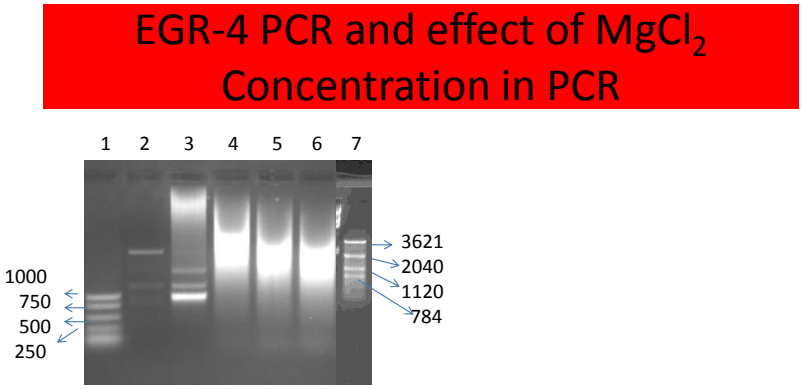


	1: Middle Range DNA Ladder	2: 357 dN6 and rPCR	3: 303 dN6 and rPCR
Band #	MW (bp)	MW (bp)	MW (bp)
1	5000	735.8	1518.1
2	2000		837.7
3	850		
4	400		

Sample gel picture for 357 and 303 rPCR products

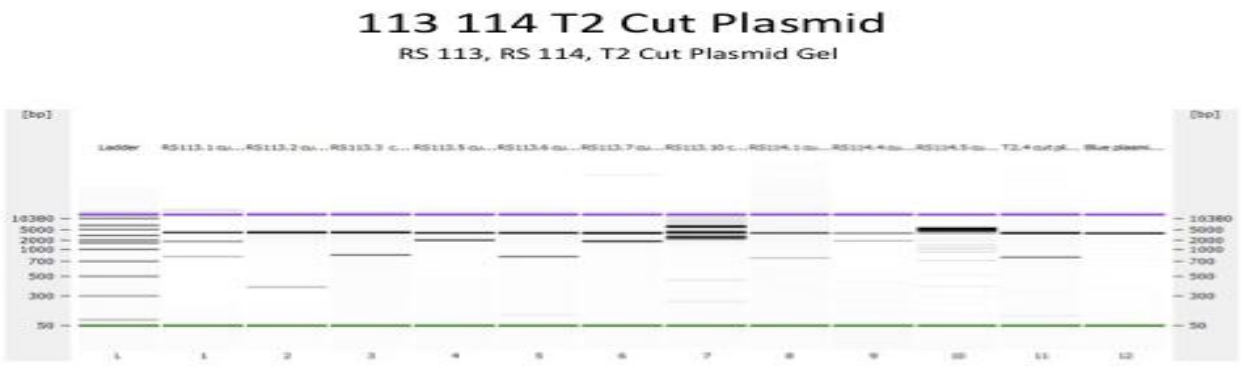
Sample	303	357	386	RS113	RS114	EGR 4	T2	TE2-4	MC35B	RS29
Sizes (bp)	1518.1	735.8	904	1930.2	2240.6	3300	1160.6		2330.1	2300
	837.7				1956.1	1672			1016.7	1000
					1614.5				596.2	
					1111.8					
					729					

Figure 10: R-PCR products generated using uniquely designed random primer-dN_6 sequence as shown in the Figure 1



Ethidium Bromide stained gel of PCR products generated from rPCR. The sizes of molecular weight markers are shown left of lane 1 molecular weight marker, lane 2 and 3 represent PCR products from 2.0 and 2.5, 3.0, 3.5 and 4.0 mM concentration of $MgCl_2$ in the PCR reaction mixture.

Figure 11: Optimum PCR conditions for cDNA synthesis and PCR amplification



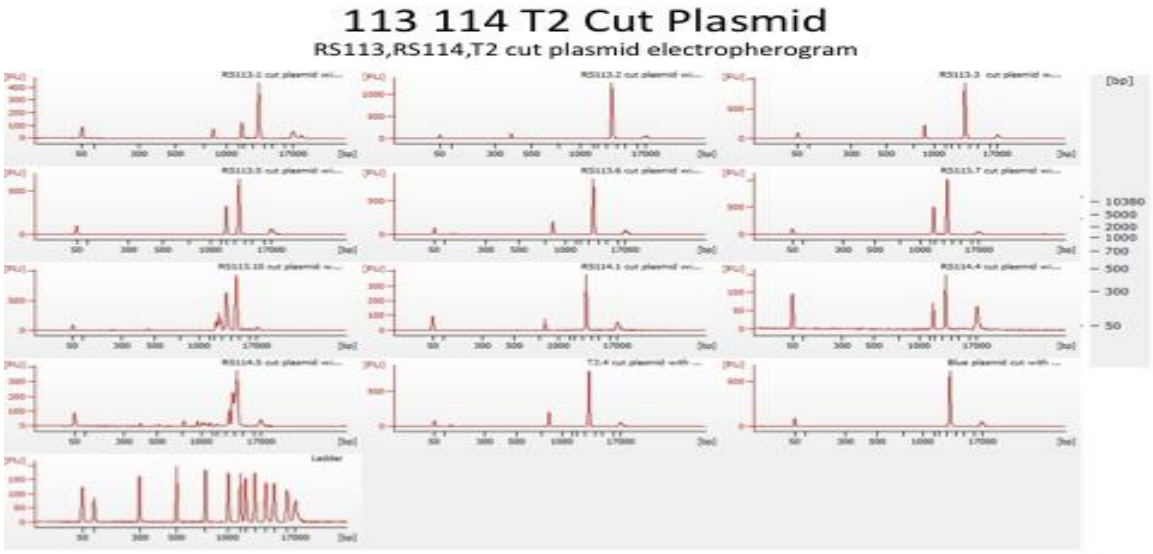
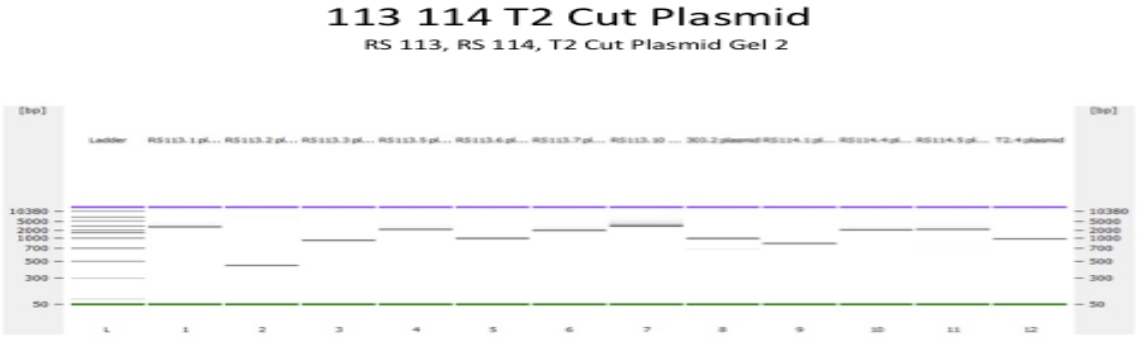


Figure 12: Gel Electrophoresis of fast digest cloned plasmids from the isolates 113, 114, and T2 dsRNA. The bottom panel shows the electrpherogram of the cloned fragments from representative clone analyzed.



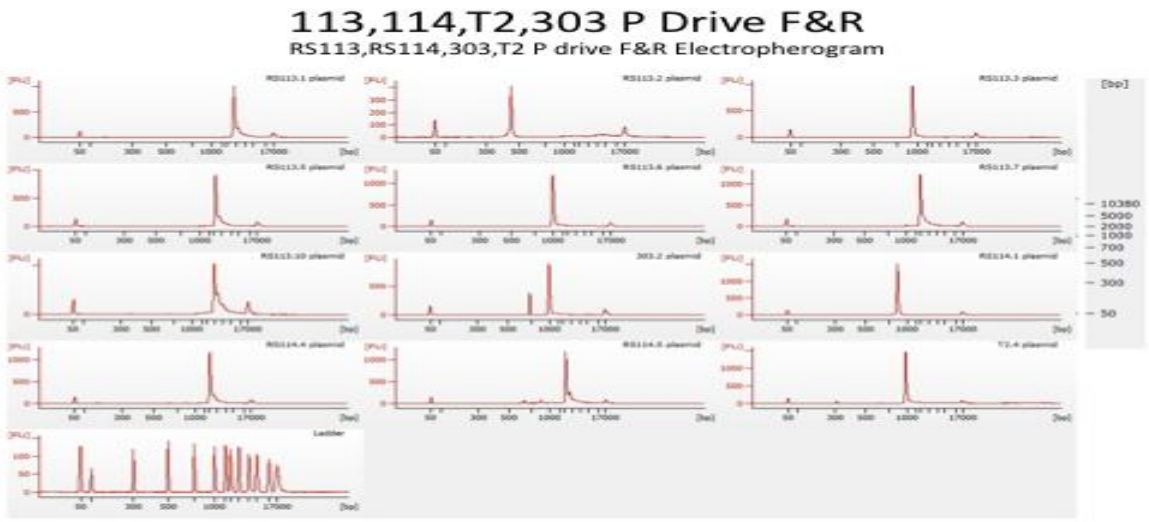
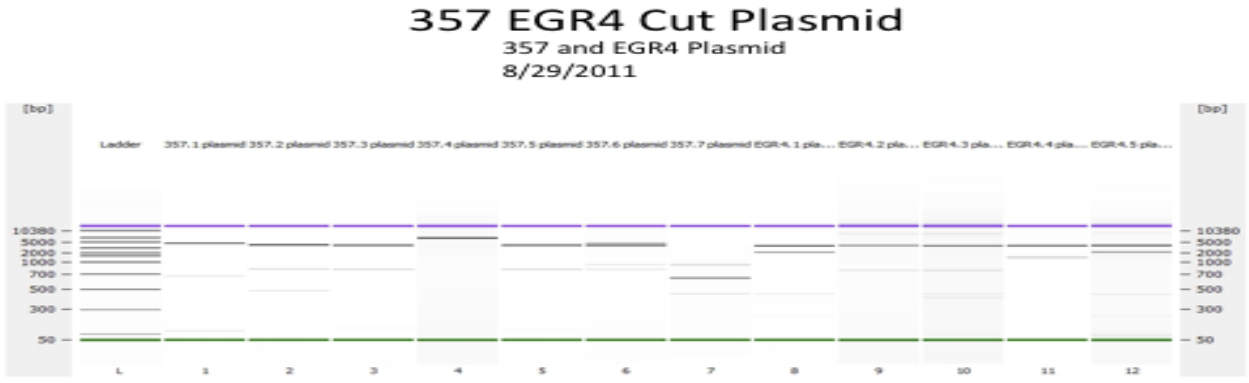


Figure 13: Gel Electrophoresis of fast digest cloned plasmids from the isolate 113, 114, T2, and 303 . The bottom panel shows the electrpherogram of the cloned fragments from representative clone analyzed



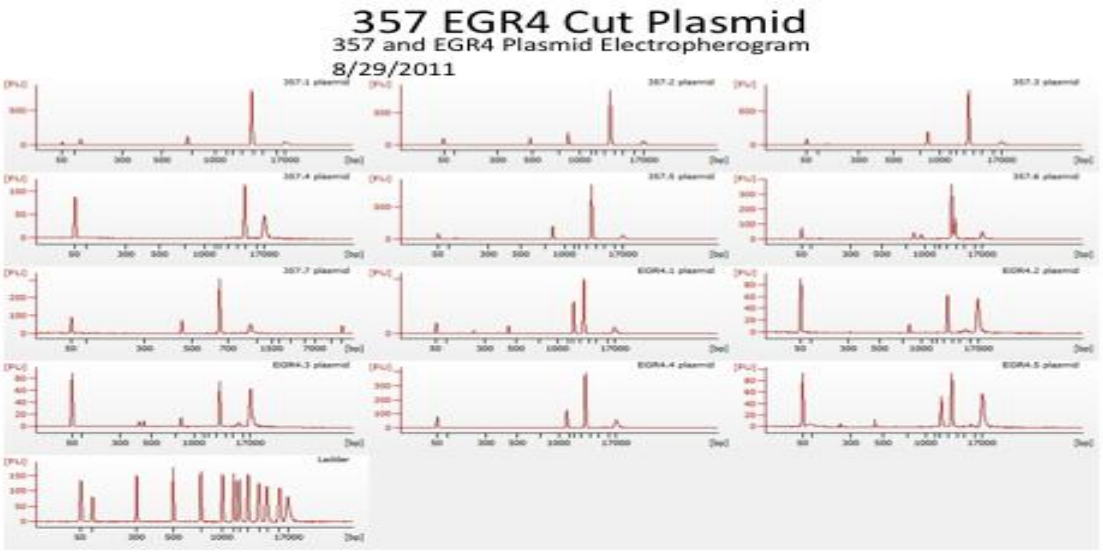


Figure 14 : Gel Electrophoresis of fast digest cloned plasmids from the isolate 357 and EGR-4. The bottom panel shows the electrpherogram of the cloned fragments from representative clone analyzed.

Primer Probe Design from Cloned dsRNA Fragments from the isolates 303, 357, 386, EGR-4 and T2 Containing R. solani Virus Isolates

Table 3: Forward and Reverse primer design

Culture	dsRNA size (bp)	dN6/rPCR size	Forward sequence	Revese sequence	Probe sequence	F&R PCR
TOMV	6000	407.7	TCCGGAAACTCACAACCCTTTG	GCTGCATGTTTGGCTTCGAT	AAGTACAGCAGACTGTCGTCACCGC	
303	1966	1518				
357	1970	736	TTCCTCCTGGAGCAGTCAATC	GGCGCTGAGGTAGAGTTGATC	CCGTACCCGGCAGACTGTTTCTA	
386	1914	904				
EGR4	3200		AGCGCTGACCTTGCTATCGAATC	CCACCGGAAGAGGGAAATCC	AGTGCCGATCAGCCCTCCACCG	
T2	2692	1160.6	TTCCTCCTGGAGCAGTCAATC	GGCGCTGAGGTAGAGTTGATC	CCGTACCCGGCAGACTGTTTCTA	

Table 4: Reaction Volume

Reagent	Volume (µL)
2X SensiFAST Probe One-Step Mix	10
Forward Primer (10 uM)	0.8
Reverse Primer (10 uM)	0.8
Probe (10 uM)	0.2
Reverse transcriptase	0.2
RiboSafe RNase Inhibitor	0.4
Water	3.6
Template (plasmid)	4
Final Volume	50

Table 5: Reaction Volume

Reagent	Volume (µL)
2X Sensi Mix II Probe	25
Forward Primer (5 uM)	2
Reverse Primer (5 uM)	2
Probe (5 uM)	0.5
Water	15.5
Template (plasmid)	5
Final Volume	50

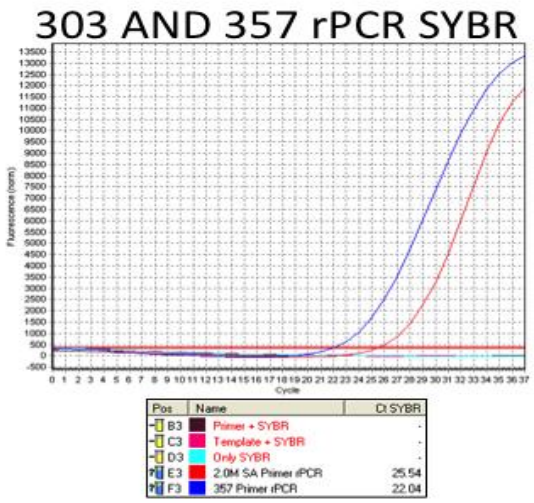


Figure 15: Real-Time PCR analysis using primers specifically obtained from the isolate 357.

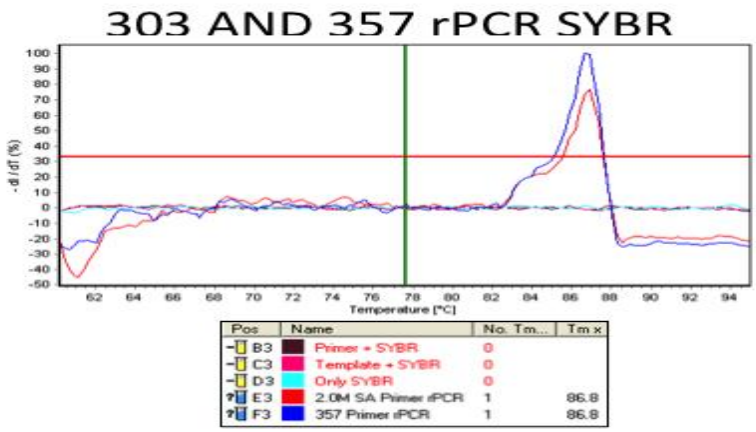


Figure 16: Melt curve analysis of the PCR product generated from the reaction in Figure 15.

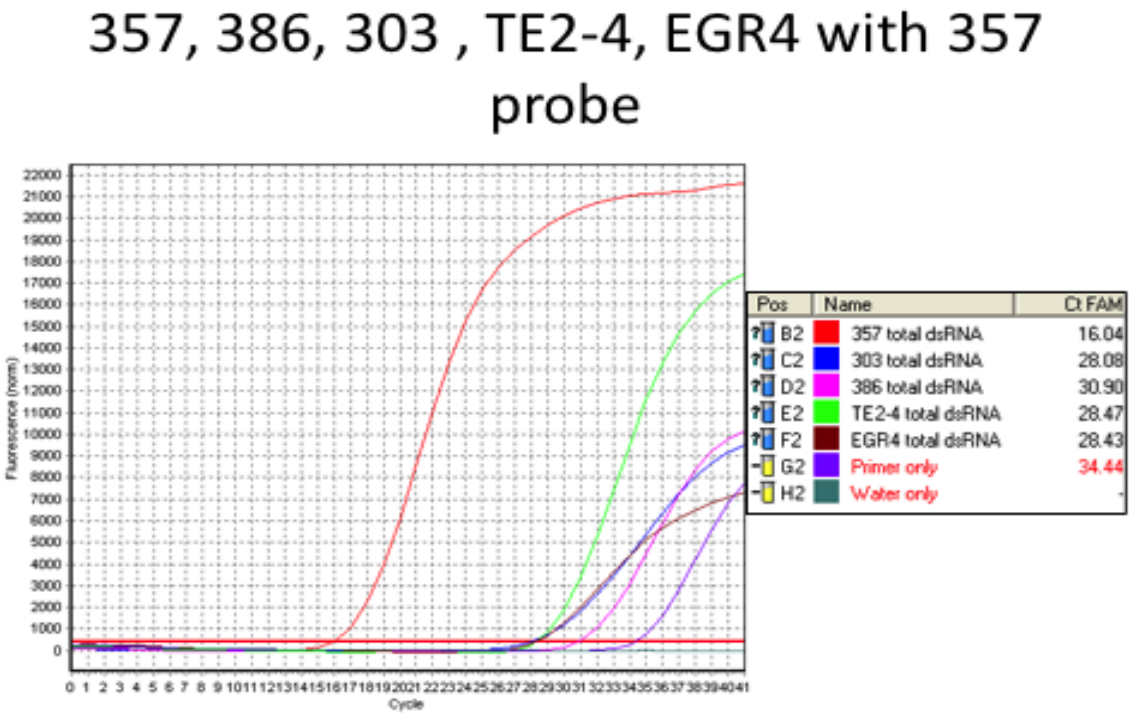
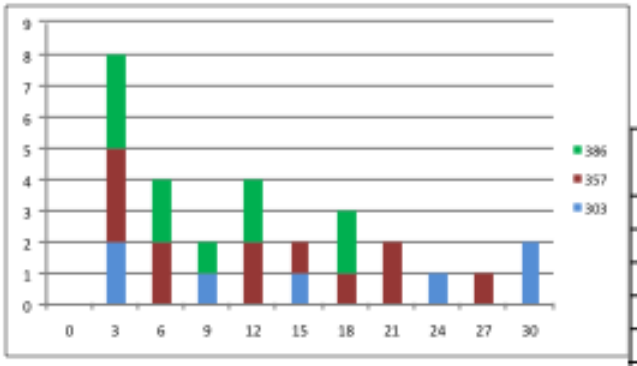


Figure 17: Real-Time PCR analysis using primers specifically obtained from the isolate 357 and probes designed from 357.

Real-Time PCR Analysis Forward and Reverse Primers of Virus from the Isolate 357

Primer Sequence: Forward: CATGCTGCAGACGCGTTA
Reverse: AAGCTTGTCGACGAATTCAGA



Primer: 29.33

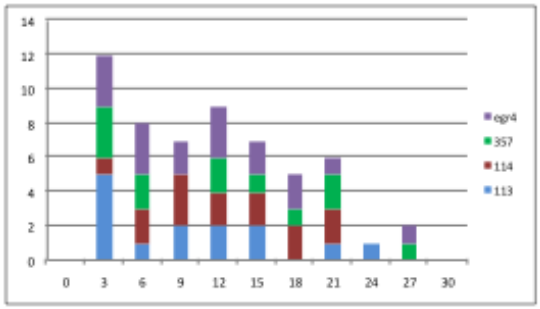
Ct value	# of clones	# of clones
0-3	0	0
3.01-6	8	8
6.01-9	4	12
9.01-12	2	14
12.01-15	4	18
15.01-18	2	20
18.01-21	4	24
21.01-24	2	26
24.01-27	6	32
27.01-30	16	48
30.01-33	6	54

cT value	maximum	minimum
Primer: 29.33	30.31	3.57

Figure 18: Real-Time PCR analysis of target nucleic acid from three virus isolates 303, 357, and 386. The primer sequence was designed from the isolate 357.

Real-Time PCR Analysis Forward and Reverse Primers of Virus from the Isolate 357

Primer Sequence: Forward: CATGCTGCAGACGCGTTA
Reverse: AAGCTTGTCGACGAATTCAGA



Isolates	Primer 357
EGR-4	17 clones tested
114	14 clones tested
357	12 clones tested
113	14 clones tested

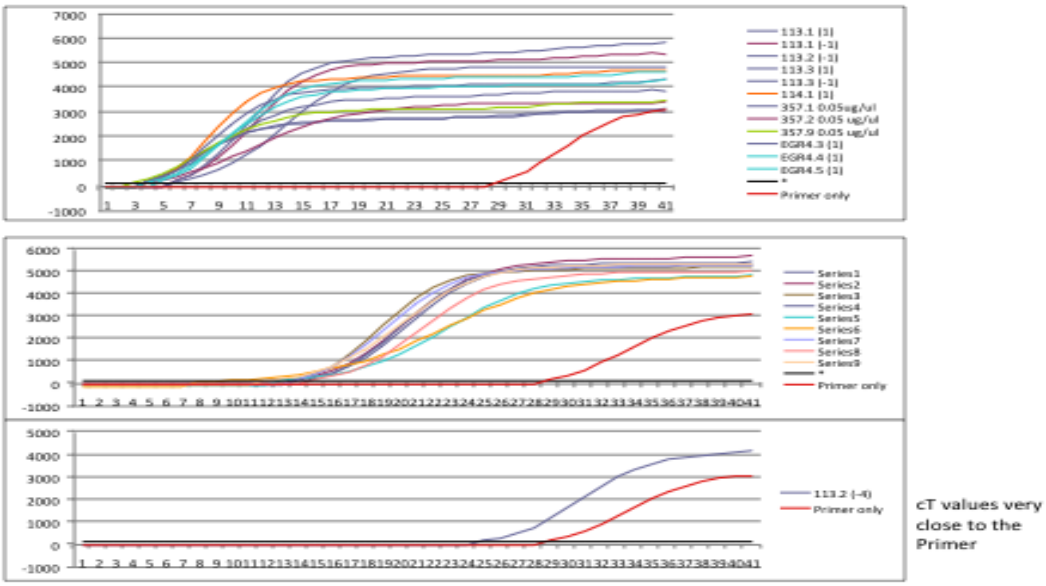


Figure 19: Real-Time PCR analysis of target nucleic acid from three virus isolates EGR-4, 357, 114, and 113. The primer sequence was designed from the isolate 357.

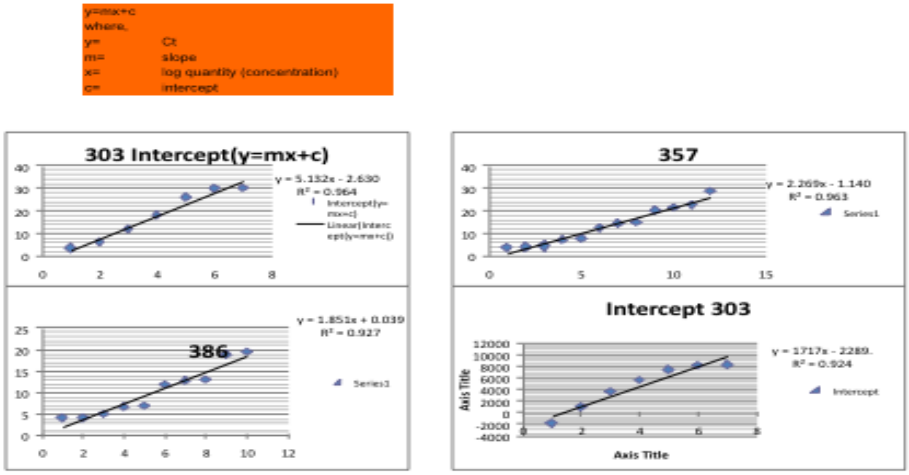


Figure 20: Bioinformatics of the Real-Time PCR data using specific primers sequences and target nucleic acid from 303, 386, and 357.

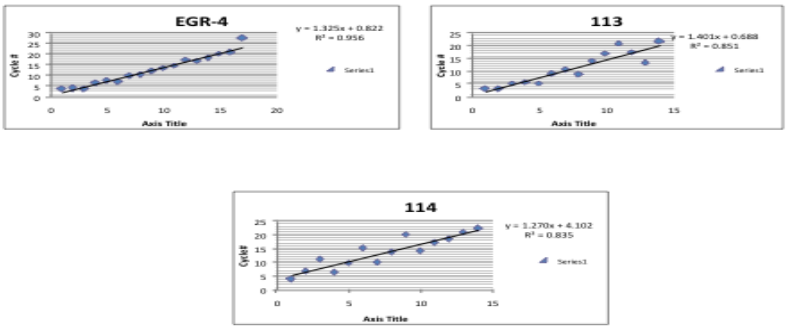


Figure 21: Bioinformatics of the Real-Time PCR data using specific primers sequences and target nucleic acid from EGR-4, 113, and 114.

Objective:

To test the variations in Ct values with the increase in the annealing temperature

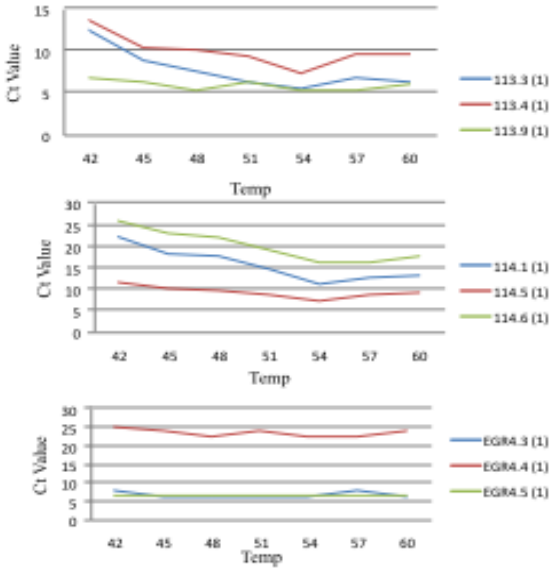


Figure 22: Results from comparison various annealing temperatures of the forward and reverse primers to the target nucleic from 113, 114, and EGR-4

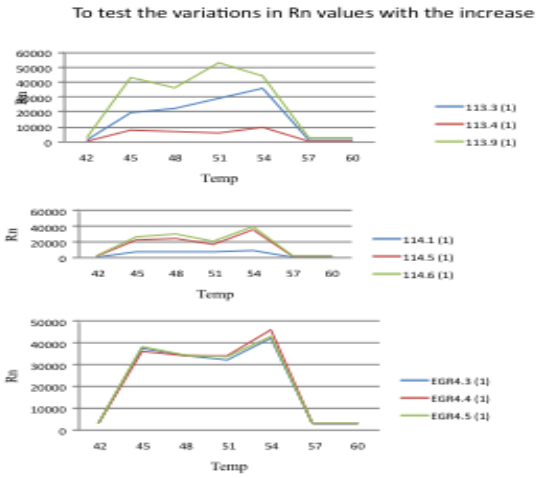
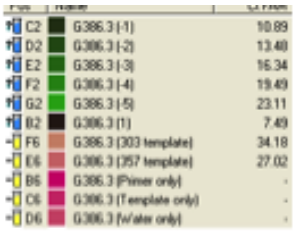


Figure 23: Effect of increase in annealing temperatures of the primers to target nucleic acid on Rn values from isolates 113, 114, and EGR 4.



Symbols	Concentration
(1)	0.5 ug
(-1)	X 10^{-1}
(-2)	X 10^{-2}
(-3)	X 10^{-3}
(-4)	X 10^{-4}
(-5)	X 10^{-5}

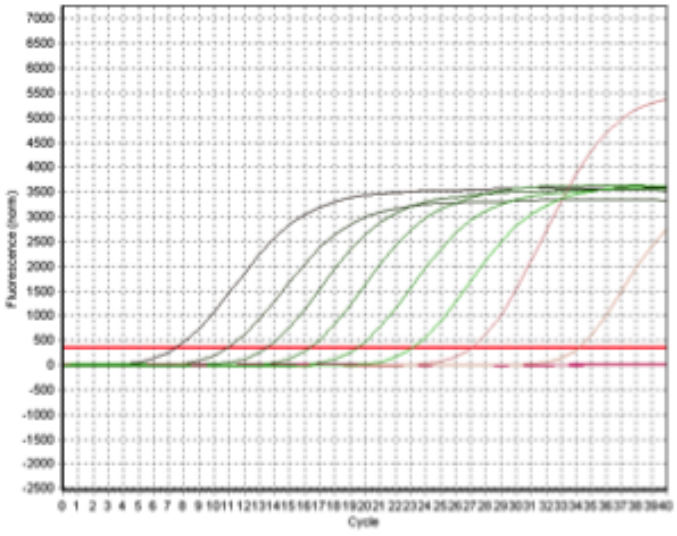


Figure 24: Probe from the virus isolate 386 and target nucleic 386 (limit of detection) using various dilutions

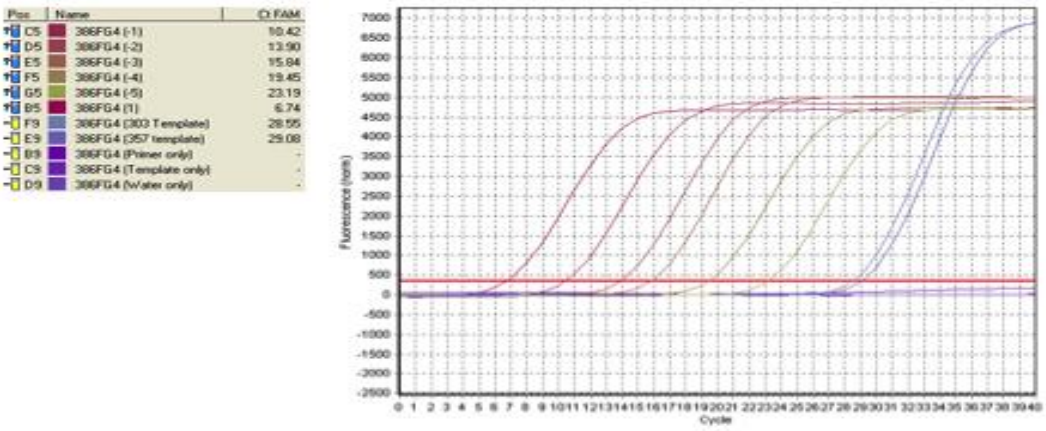


Figure 25: Probe from the virus isolate 386 and target nucleic (limit of detection) using various dilutions of the target dsRNA nucleic acid

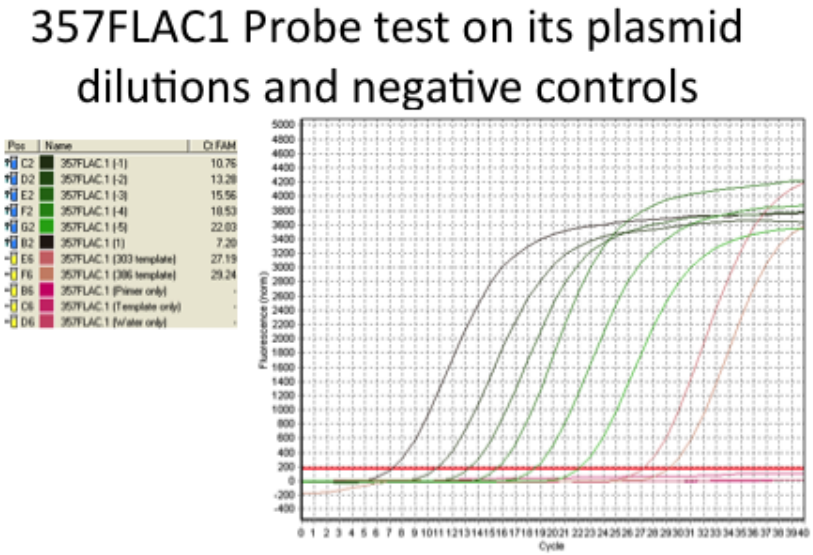


Figure 26: Probe from the virus isolate 357 and target nucleic (limit of detection) using various dilutions of the target dsRNA nucleic acids.

Figure 27: Probe from the virus isolate 303 and target nucleic (limit of detection) using various dilutions of the target dsRNA nucleic acids.

Figure 27: Probe from the virus isolate 303 and target nucleic (limit of detection) using various dilutions of the target dsRNA nucleic acids.

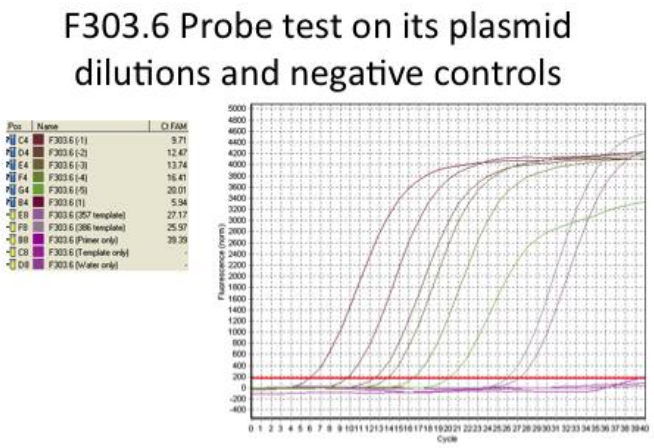


Figure 27: Probe from the virus isolate 303 and target nucleic (limit of detection) using various dilutions of the target dsRNA nucleic acids.

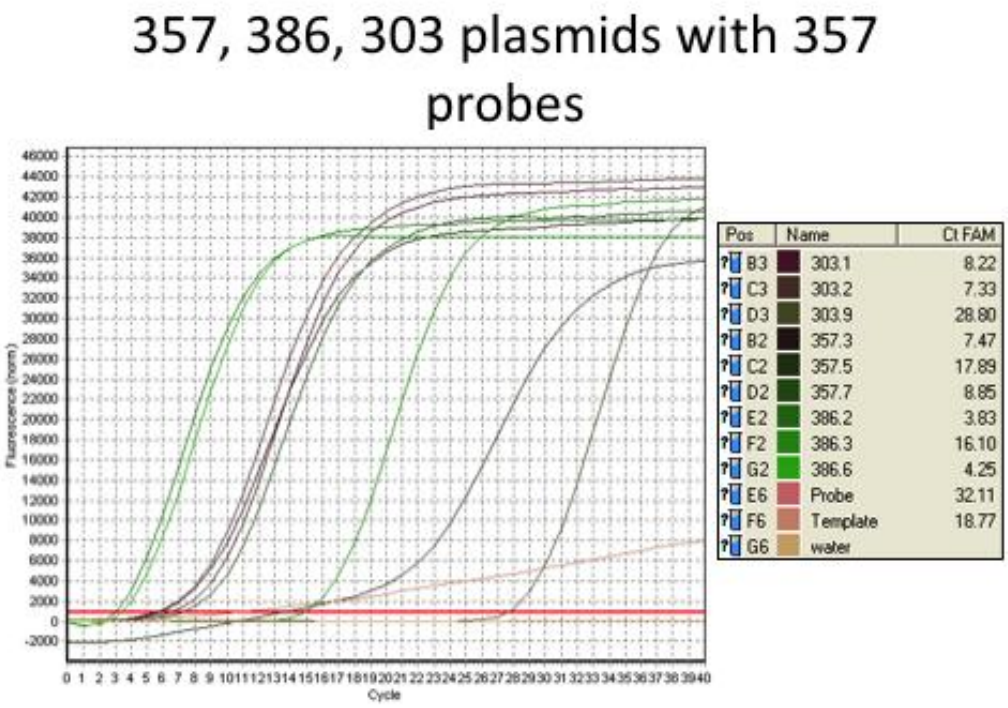


Figure 28: Probe from the virus isolate 357 and target nucleic acids coming from different targets (limit of detection) using various dilutions of the target dsRNA nucleic acids.

Appendix C

Milestone 2:

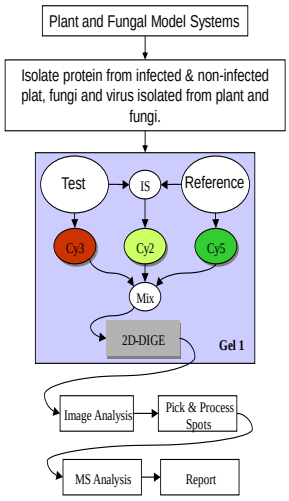
Task 2:

Table 1: Fungal isolates tested for Proteomic Analysis

Fungal Isolate Name	Growth Characteristics	Type of Nuclei	Genome Type
RS 303	Rapid Growth; Brown	Heterokaryon	Normal
RS 357	Rapid Growth; Brown	Heterokaryon	Normal
RS 386	Slower Growth; Brown	Heterokaryon	Normal
Rhs 113	Slower Growth; Brown	Heterokaryon	Normal
Rhs 114	Slower Growth; Brown	Heterokaryon	Normal
TE2-4	Rapid Growth; Downy	Heterokaryon	Normal
RS 29	Slower Growth; Pale yellow	Heterokaryon	Normal
RS29.3	Slower Growth; White and Downy	Homokaryon	Reduced Genome
T2	Slower Growth and white	Homokaryon	Reduced Genome
TOM-7	Rapid Growth and Brown	Homokaryon	Reduced Genome
EGR-4	Rapid Growth; white and Downy	Homokaryon	Reduced Genome
HUD 2-1	Brown and Rapid Growth	heterokaryon	Normal

Note: Heterokaryon isolates have 2 nuclei; whereas Homokaryon isolates have one nucleus

The 2-D work-flow process for protein target identification is shown in Figure 1 below.



2D-DIGE Workflow process for identification of protein targets in plant and fungal model systems

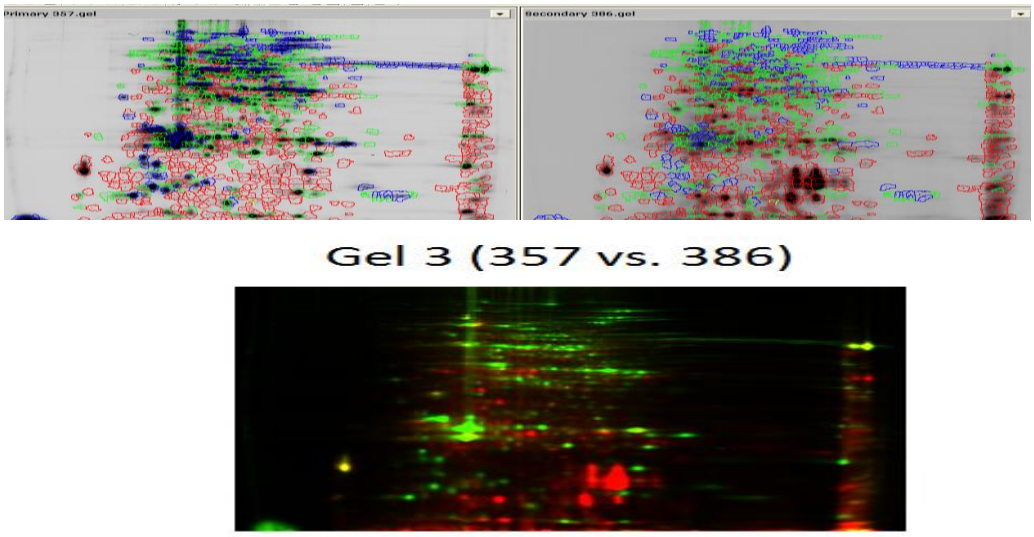
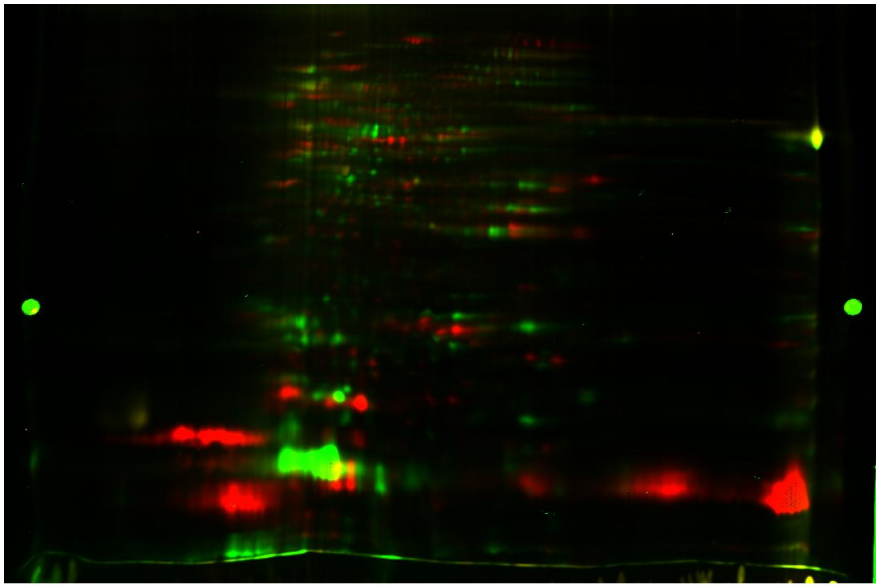


Figure 2 and 3: Representative 2D-DIGE gels showing protein portrait of two *R. solani* anastomosis groups. [A] ESNLG (GREEN spots) - EQRACH (RED spots) [B] EQRCHF50 (GREEN spots) - EQRIVEA-A51 (RED spots). Over 2400 candidate protein spots are detected on each gel

Table 2. Spot statistics of proteins in Isolates of R. solani belonging to the same AG

Total number of spots	Spot Statistics		
1310	Decreased	Similar	Increased
	526 (40.2%)	70 (5.3%)	714 (54.5%)

RS 113 and T2



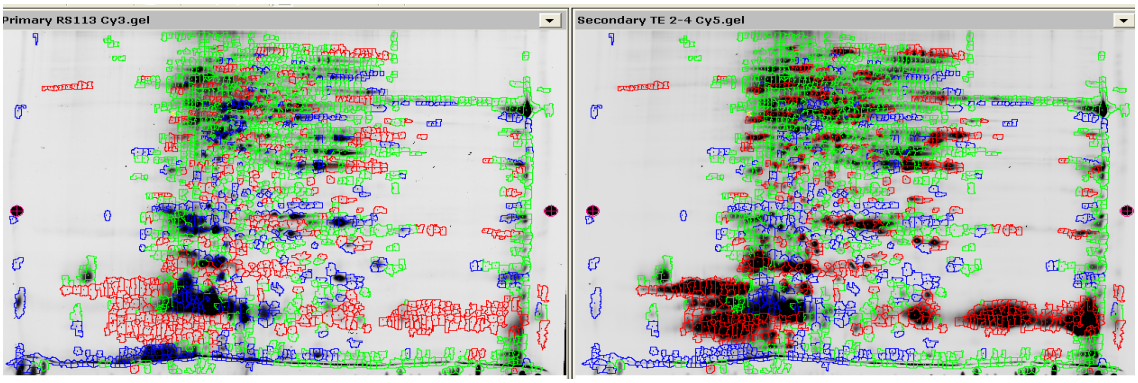
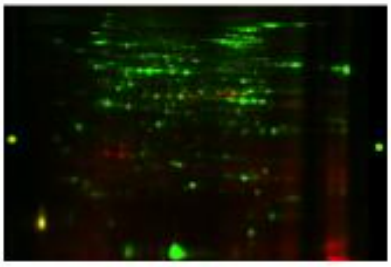


Figure 4and 5: Representative 2D-DIGE gels showing protein portrait of two *R. solani* anastomosis groups. [A] ESNLG (GREEN spots) - EQRACH (RED spots) [B] EQRCHF50 (GREEN spots) - EQRIVEA-A51 (RED spots).

Table 3. Spot statistics of proteins in Isolates of R. solani belonging to different AG

Total number of spots	**Spot Statistics**		
2126	Decreased	Similar	Increased
	413 (19.4%)	1140 (53.6%)	573 (27.%)

Spots Differentially Expressed 2-fold or Higher
29.7 vs. RS29

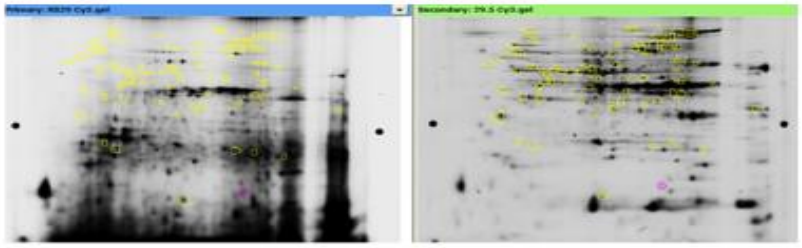


Green-Indicates over expressed proteins in 29.7 (Cy3)

Red- Indicates over expressed proteins in RS29 (Cy5)

Spot Statistics-2-fold or greater spot ratio threshold	
Similar	1403(90.4%)
Increased in 29.5	114(7.3%)
Decreased in 29.5	35(2.3%)

29.5 vs. RS29
Unique Spots



Number of spots in 29.5 only	88
Number of spots in RS29 only	10

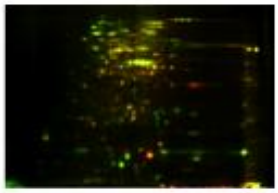
Figure 6 and 7: Comparison of differentially expressed protein spots on the wild type (RS 29) and reduced genome complement isolate (29.5).

29-3A vs. RS29

2 Unique protein spots found

Total Number of Spots	Spot Statistics		
1751	Decreased	Similar	Increased
	167,[9.5%]	1402, [80.1%]	182, [10.4%]

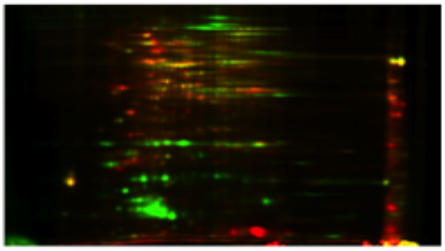
RS29 vs. 29-3A



No unique protein spots found

Total Number of Spots	Spot Statistics		
1438	**Decreased**	**Similar**	**Increased**
	150,[10.4%]	1163, [80.9%]	125, [8.7%]

Figure 7 and 8: Up-regulated and down regulated proteins in (29.3A) reduced genome complement isolate and wild type RS 29.



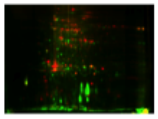
T2 vs. TOM7

51 Unique protein spots found

Total Number of Spots	Spot Statistics		
2221	**Decreased**	**Similar**	**Increased**
	665,[29.9%]	1055, [47.5%]	501, [22.6%]

Figure 9: Up-regulated and down regulated proteins when T2 was compared against TOM 7 both have reduced genome complement isolates.

TOM7 vs. T2

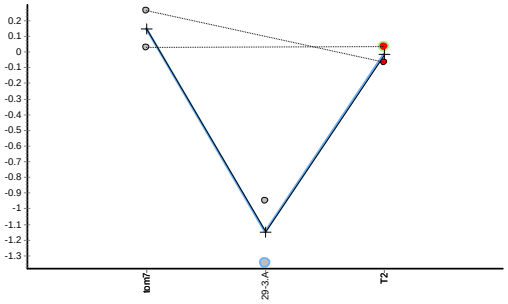


75 Unique protein spots found

Total Number of Spots	Spot Statistics		
1628	**Decreased**	**Similar**	**Increased**
	541,[33.2%]	562, [34.5%]	525, [32.2%]

Figure 10: Up-regulated and down regulated proteins when TOM7 was compared against T2both have reduced genome complement isolates.

Graph view 29-3.A vs. TOM7
(Down regulated)



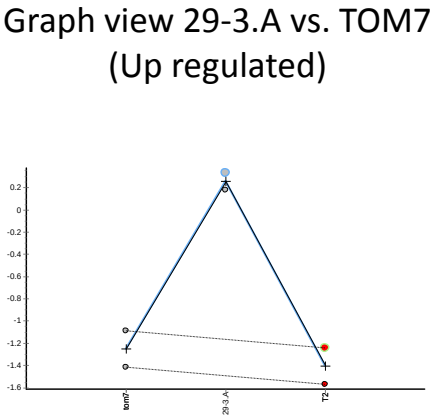
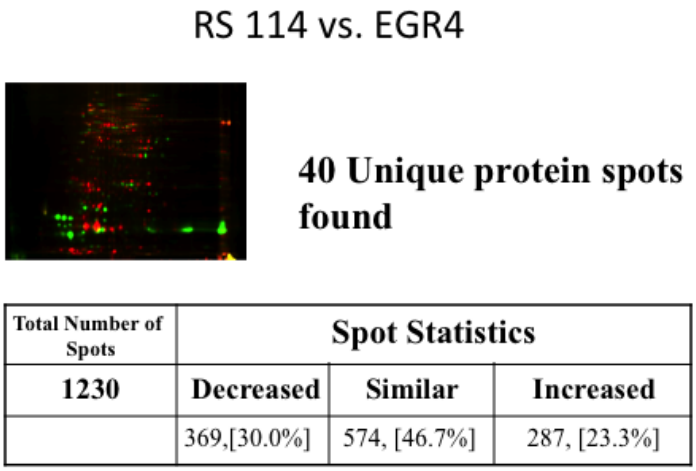


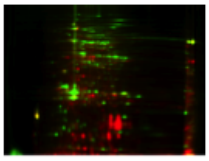
FIGURE : 11 Graphic view of protein regulation in selected isolates of *R.solani isolate 29.3 and TOM7*



Total Number of Spots	Spot Statistics		
1230	Decreased	Similar	Increased
	369,[30.0%]	574, [46.7%]	287, [23.3%]

Figure 12: Representative 2D-DIGE gels showing protein portrait of two *R. solani* isolates wild type heterokaryon (RS 114) having the virus and reduce genome complement also having the virus (EGR-4) [A] ESNLG (GREEN spots) - EQRACH (RED spots) [B] EQRCHF50 (GREEN spots) - EQRIVEA-A51 (RED spots).

357 vs. 386

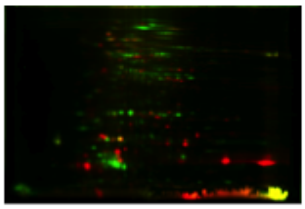


20 Unique protein spots found

Total Number of Spots	Spot Statistics		
1310	Decreased	Similar	Increased
	526,[40.2%]	70, [5.3%]	714, [54.5%]

Figure 13: Representative 2D-DIGE gels showing protein portrait of two *R. solani* isolates wild type heterokaryons (357) having the virus and 386 also heterokaryon and having the virus [A] ESNLG (GREEN spots) - EQRACH (RED spots) [B] EQRCHF50 (GREEN spots) - EQRIVEA-A51 (RED spots).

HUD2-1 vs. WT 303

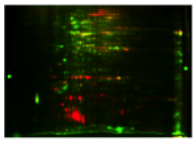


49 Unique protein spots found

Total Number of Spots	Spot Statistics		
1393	Decreased	Similar	Increased
	380,[27.3%]	644, [46.2%]	369, [26.5%]

Figure 14: Representative 2D-DIGE gels showing protein portrait of two *R. solani* isolates wild type heterokaryons (303) having the virus and HUD2-1also heterokaryon and but not having the virus [A] ESNLG (GREEN spots) - EQRACH (RED spots) [B] EQRCHF50 (GREEN spots) - EQRIVEA-A51 (RED spots).

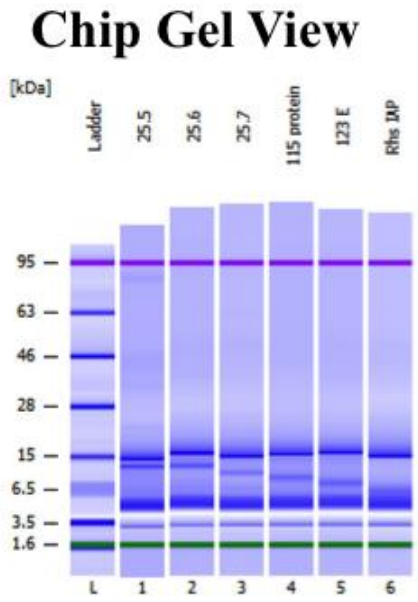
386 vs. 357



25 Unique protein spots found

Total Number of Spots	Spot Statistics		
1854	**Decreased**	**Similar**	**Increased**
	348,[18.8%]	1002, [54.0%]	504, [27.2%]

Figure 15: Representative 2D-DIGE gels showing protein portrait of two *R. solani* isolates wild type heterokaryons (386) having the virus and 357-also heterokaryon and having the virus [A] ESNLG (GREEN spots) - EQRACH (RED spots) [B] EQRCHF50 (GREEN spots) - EQRIVEA-A51 (RED spots).



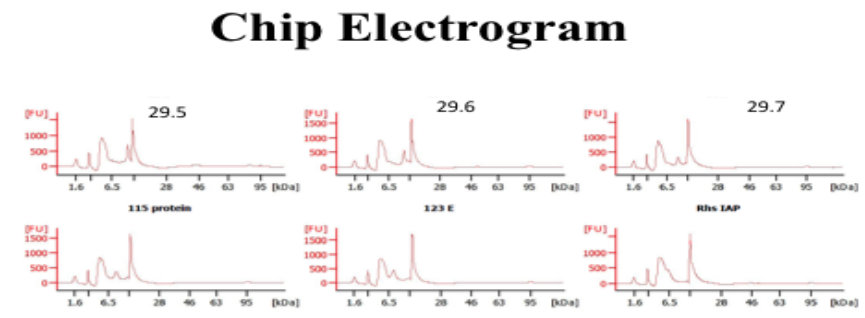


Figure 16: Representative Protein sample on Agilant bio-analyzer showing protein portrait of *R. solani* isolates one wild type heterokaryons (Rhs1AP) having the virus and all others that are virus free and reduce genome complements.

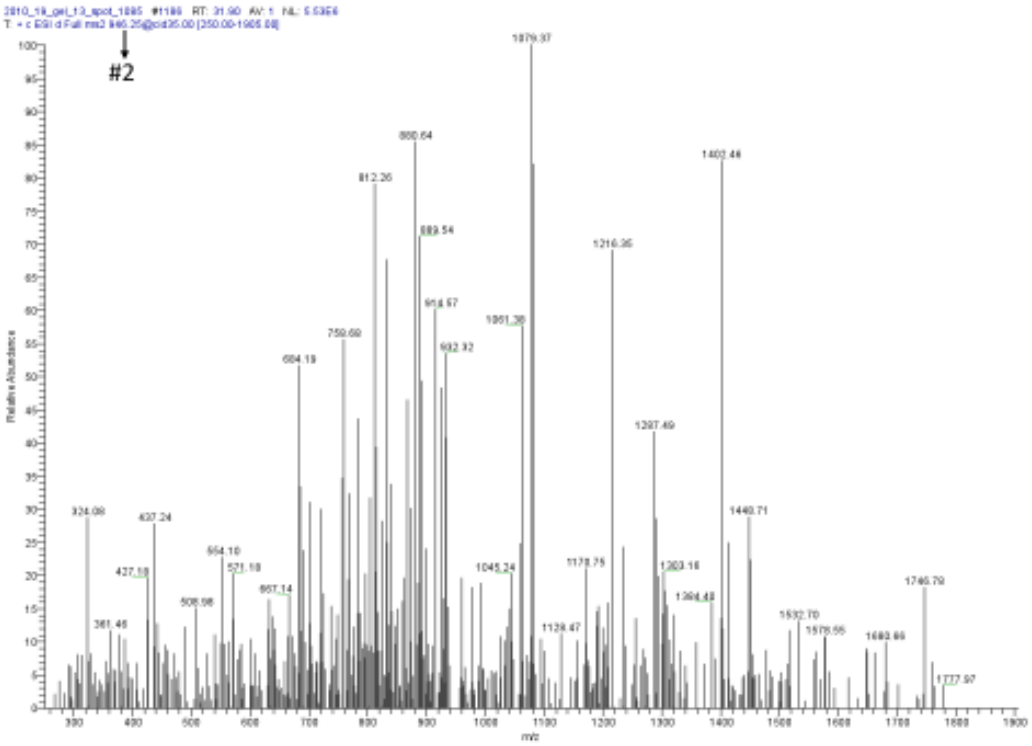


Figure 16: peptide sequencing where a full MS scan from m/z 400-1500 is followed by MS/MS scans on the five most abundant ions.

Appendix D

MILESTONE 3

TASK 1:

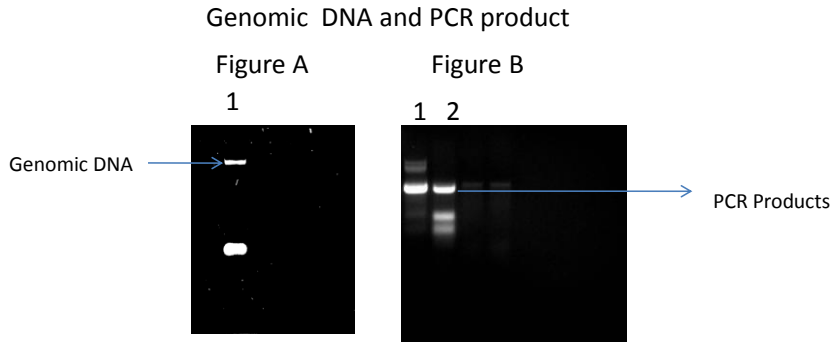


Figure A: Lane 1 Purified Genomic DNA
Figure B: Lanes 1 and 2 PCR products from Genomic DNA

Table 1: *R. solani* isolate summary of peptide sequence and related protein products

Protein Sequence	Accession number	Characteristics
TMSDLELENSLDEALILFR	257735910	Unnamed protein product (Ustilago maydis 521)
KACDDLGANRYGDELGR	71019591	Hypothetical protein UM03879.1(Ustilago maydis 521)
QSGLHPRGVGTGGAASDENAKGK	71004496	Hypothetical protein UM00767.1 (Ustilago maydis 521)
YPKNELDVGGTVPWYSILTK	342882884	Hypothetical protein FOXB-06029 (Fusarium oxysporum F05176)
AWSADFLAKKPELAAHLAQR	71003335	Hypothetical protein UM00201.1 [Ustilago maydis 521]
EAEASKVDGDGDVGMEDSAAPEETTIKK	342883340	hypothetical protein FOXB_05636 [Fusarium oxysporum Fo5176]

Table 2: Protein spot Characteristics from select Isolates of *R. solani*

Spot No 706	**Present Only in RH3**
257735910	unnamed protein product [Ustilago maydis 521]
71019591	hypothetical protein UM03879.1 [Ustilago maydis 521]
Spot No 724	**Present Only in RH3**
342867753	hypothetical protein FOXB_16946 [Fusarium oxysporum Fo5176]
342878479	hypothetical protein FOXB_09675 [Fusarium oxysporum Fo5176]
Spot No 1406	**Present Only in RH3**
71004496	hypothetical protein UM00767.1 [Ustilago maydis 521]
342882884	hypothetical protein FOXB_06029 [Fusarium oxysporum Fo5176]
Spot No 1126	**Present Only in RS29**
71003335	hypothetical protein UM00201.1 [Ustilago maydis 521]
342883340	hypothetical protein FOXB_05636 [Fusarium oxysporum Fo5176]
Spot No 1169	**Present Only in RS29**
342884726	hypothetical protein FOXB_04497 [Fusarium oxysporum Fo5176]
71022051	hypothetical protein UM05109.1 [Ustilago maydis 521]
Spot No 1277	**Present Only in RS29**
269979980	trichodiene synthase [Fusarium incarnatum]
74702006	Glucose-repressible alcohol dehydrogenase transcriptional effector

Procedure for Nuclear Staining and Hyphal Anastomosis

FIRST DAY

- Prepare each isolate on a Petri-dish containing 20 ml of potato dextrose agar (PDA) and incubate 2 days.

SECOND DAY

- Soak glass slides in plastic box filled with 70 % ethanol for sterilizing **(Fig. A)**.

THIRD DAY

- Dry glass slides putting on paper towel **(Fig. B).**
- Write the names of pairing isolates on the edge of each glass slide.
- Wipe up inside of the plastic box.

Fig. A

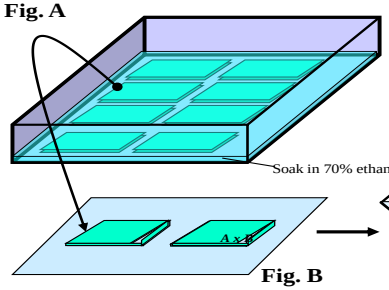


Fig. B

- Prepare 60 ml of 2% water agar (WA) in 100 ml bottle.
- Using forceps, dip each glass slide in WA **(Fig C).**
- Wipe the under side of slide with sterile paper towel to remove agar **(Fig D)**.

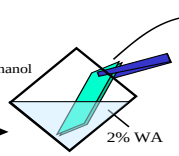


Fig. C

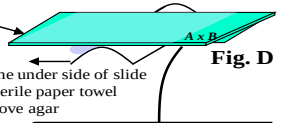


Fig. D

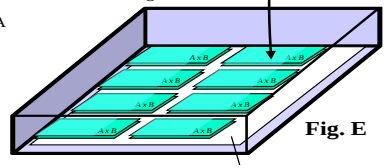


Fig. E

- Set the paper towel on the plastic box and moisturized by distilled water, and put the glass slides into box **(Fig E)**.
- Transfer two agar plugs from growing mycelium on PDA to WA on glass slide **(Fig F).**
- Place plugs 2-3 cm agar and prepare two replicates of each isolate including self control pairings.
- Cover and incubate for 12 to 48 hours at 25 C in the dark.

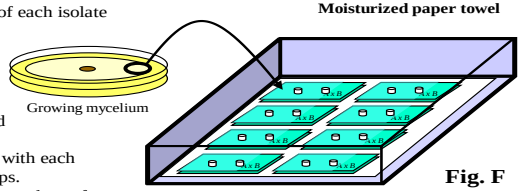


Fig. F

FOURTH or FIFTH DAY

- Prepare 0.1% phenosafranin (10 mg/10 ml H_2O) and 3% KOH (0.3 g/10 ml H_2O) staining solutions.
- When mycelium of paired colonies come in contact with each other, remove agar plugs carefully with sterile forceps.
- Add one drop of 0.1% phenosafranin, and then add one drop of 3% KOH **(Fig G).**
- Cover with a clean glass cover slip and examine microscopically.

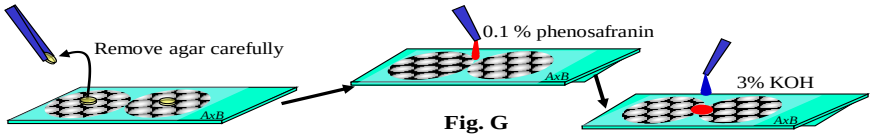


Fig. G

Figure 1: Steps in Staining the actively growing fungal hyphae

Incompatible Interaction-Panoramic Imaging

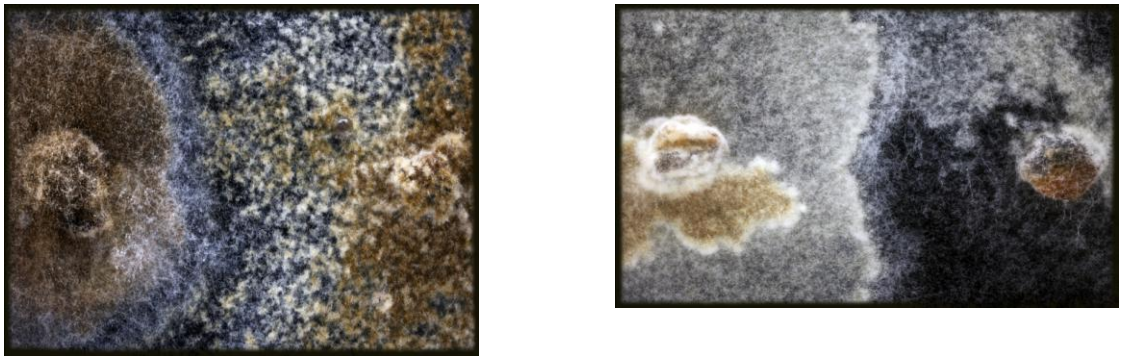


Figure 2: Fungal Interactions: Mutant and Wild type

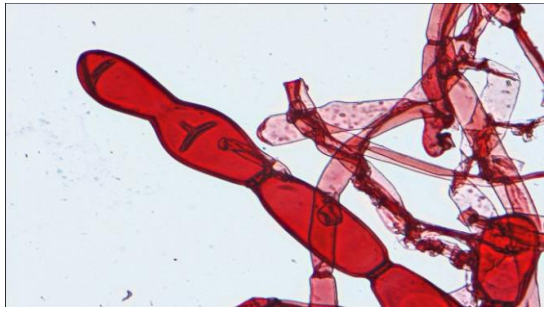


Figure 3: Incompatible Interaction

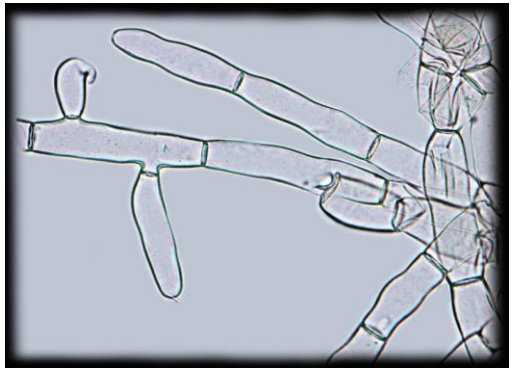


Figure 4: Compatible Interaction

Fungal Interactions at Microscopic level

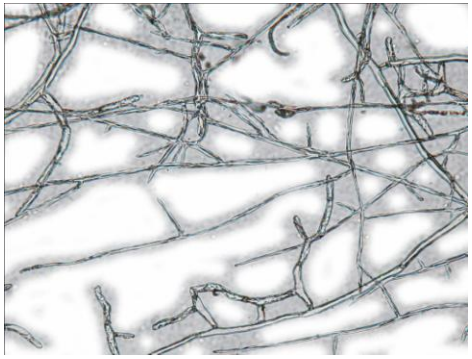


Figure 5 Killing reaction

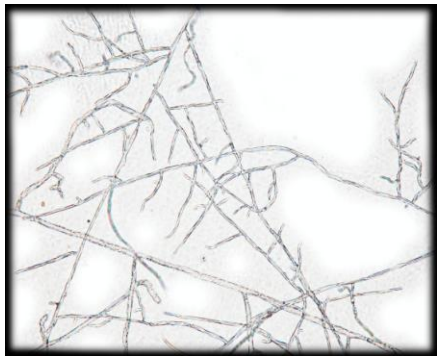


Figure 6 Compatible Interaction

Incompatibility Interactions: Time course Interactions: Several isolates

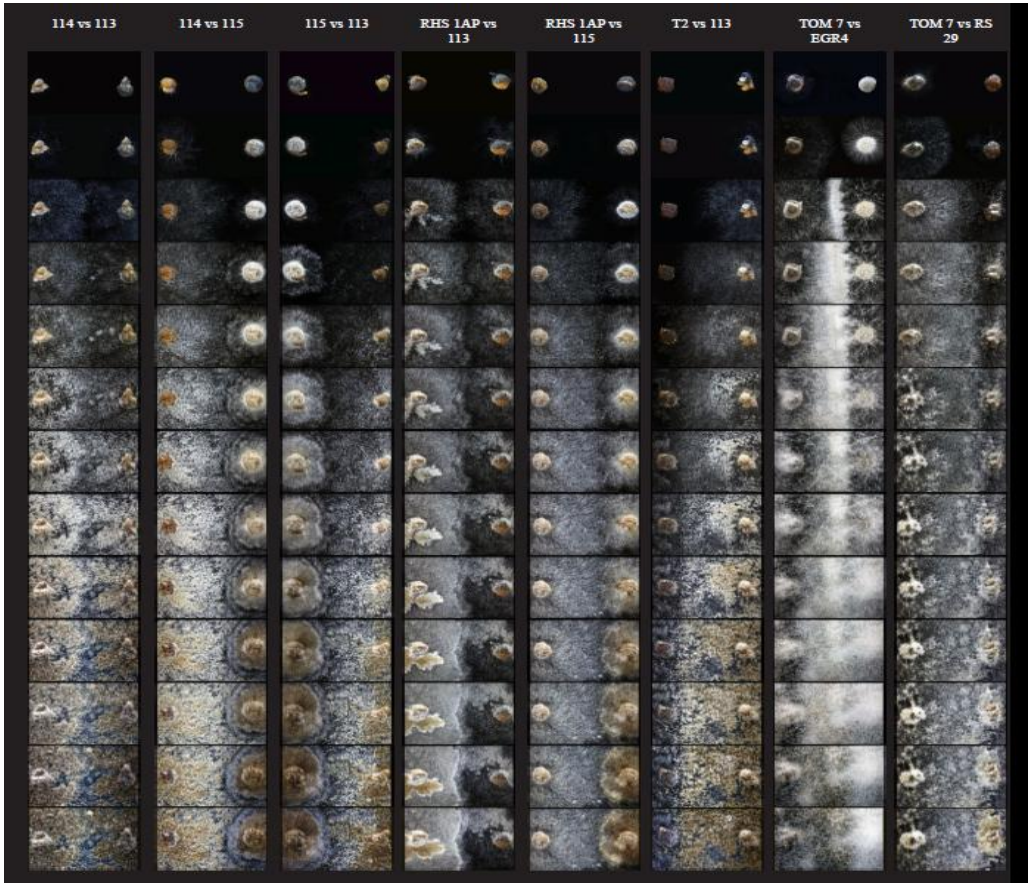
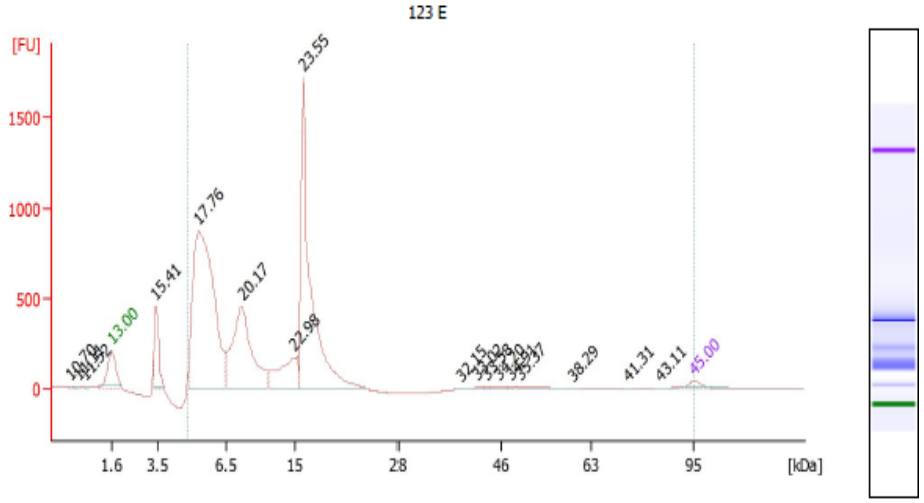


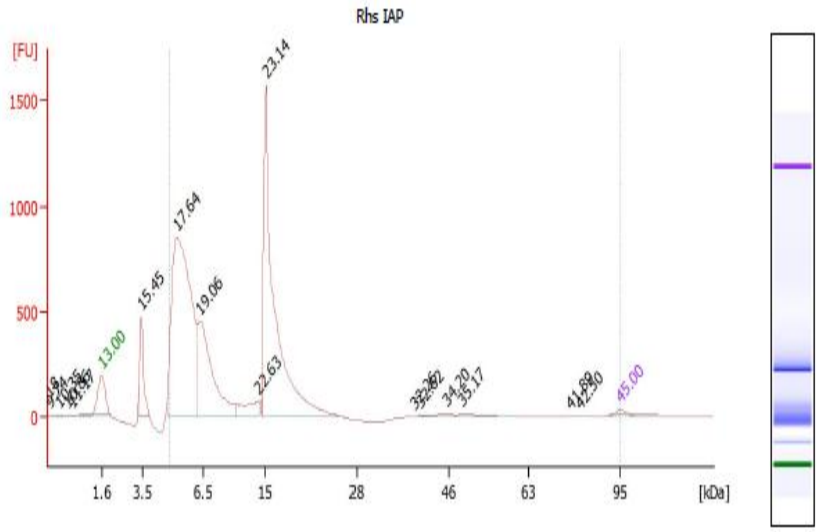
Figure 7: Fungal Interactions at Microscopic level at various time intervals

Figure 8 : Protein profile following fungal anastomosis



Overall Results for sample 5 : 123 E

Number of peaks found:	13	Noise:	1.5
Total Rel Conc:	8,895.5 ng/µl		



Overall Results for sample 6 : Rhs IAP

Number of peaks found:	10	Noise:	1.8
Total Rel Conc:	10,192.7 ng/µl		